University of Alberta

RAMP-2 DISTRIBUTION IN THE CNS AND REGULATION BY CHANGES IN BLOOD PRESSURE

by

Tevye Jason Eugene Stachniak ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Center for Neuroscience

Edmonton, Alberta

Fall 2002

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Faculty of Graduate Studies and Research

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Jack H. Jhamandas

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Sept. 27, 2002

Date

Dedication

To my family, who gave me a love of life,

And my teachers, who gave me a love of learning.

Abstract

Adrenomedullin (ADM) is a neuropeptide that acts in the central nervous system (CNS) to regulate blood pressure. An ADM receptor is composed of receptor activity modifying protein 2 (RAMP-2) and calcitonin receptor like receptor (CRLR). We used *in situ* hybridization to localize RAMP-2 mRNA, and performed RT-PCR to detect CRLR mRNA. RAMP-2 is expressed in numerous areas, including autonomic nuclei. Many regions expressing RAMP-2 mRNA also express a low level of CRLR mRNA. Next, we examined changes in the expression of RAMP-2 and preproADM in the brain in response to blood pressure manipulations. Rats received intravenous infusion of nitroprusside or phenylephrine, to decrease or increase blood pressure, respectively. Decreased blood pressure caused an increase in RAMP-2 mRNA levels in the nucleus of the solitary tract (NTS), and a decrease in preproADM signal in the paraventricular nucleus (PVN). Increased blood pressure caused a decrease in RAMP-2 signal in the PVN and NTS. The CNS distribution and modulation of ADM signaling components are consistent with the role of ADM in the regulation of autonomic function.

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LIST OF ABBREVIATIONS

4V, 4th ventricle

ACE, central amygdaloid nucleus

ACo, anterior cortical amygdaloid nucleus

ACTH, adrenocorticotropic hormone

AD, anterodorsal thalamic nucleus

ADM, adrenomedullin

AMB, ambiguus nucleus

ANS, autonomic nervous system

AP, area postrema

Aq, aqueduct (Sylvius)

Arc, arcuate nucleus

AVP, arginine-vasopressin

BL, basolateral amygdaloid nucleus

BLA, basolateral amygdaloid nucleus, anterior

BLV, basolateral amygdaloid nucleus, ventral

BMA, basolateral amygdaloid nucleus, anterior

CA 1-3, fields CA 1-3 of Ammon's horn

cAMP, cyclic adenosine 3',5'-monophosphate

cc, corpus callosum

cDNA, complementary DNA

CGRP, calcitonin gene related peptide

clPVN, caudal lateral PVN

CM, central medial thalamic nucleus

cmPVN, caudal medial PVN

CNS, central nervous system

CRF, corticotropin-releasing factor

CRLR, calcitonin receptor like receptor

Cu, cuneate nucleus

cVLM, caudal VLM

CVO, circumventricular organ

DG, dentate gyrus

DMX, dorsal motor nucleus of the vagus

DNA, deoxyribonucleic acid

dpPVN, dorsal parvocellular division of PVN

DR, dorsal raphe nucleus

DTg, dorsal tegmental nucleus

DTT, dithiothreitol

EDTA, ethylenediaminetetraacetic acid

f, fornix

gcc, genu of the corpus callosum

Gr, gracile nucleus

HDB, horizontal limb of the diagonal band of Broca

hf, hippocampal fissure

HR, heart rate

HSLAS, Health Sciences Lab Animal Services

ICV, intracerebroventricular

IML, intermediolateral cell column

IO, inferior olive

ISH, in situ hybridization

IV, intravenous

LC, locus coeruleus

LPGi, lateral paragigantocellular nucleus

LPS, lipopolysaccharide

LSD, lateral septal nucleus, dorsal

LSV, lateral septal nucleus, ventral

MAP, mean arterial pressure

MAPK, mitogen activated protein kinase

MeA, medial amygdaloid nucleus, anterior

MD, mediodorsal thalamic nucleus

MPA, medial preoptic area

mpdPVN, dorsolateral medial parvocellular PVN

mpvPVN, ventral medial parvocellular PVN

mPVN, magnocellular PVN

mRNA, messenger RNA

NO, nitric oxide

NP, sodium nitroprusside

NTS, nucleus of the solitary tract

opt, optic tract

ox, optic chiasm

OXY, oxytocin

PAMP, proadrenomedullin N-terminal 20 peptide

PB, parabrachial nucleus

PBS, phosphate buffered saline

Pe, periventricular hypothalamic nucleus

PHENYL, phenylephrine

Pir, piriform cortex

PKC, protein kinase C

pmPVN, posterior magnocellular PVN

PoPVN, paraventricular hypothalamic nucleus, posterior

PP, posterior lobe of the pituitary

ppADM, preproadrenomedullin

pPVN, parvocellular PVN

PV, paraventricular thalamic nucleus

PVA, paraventricular thalamic nucleus, anterior

PVN, hypothalamic paraventricular nucleus

PVP, paraventricular thalamic nucleus, posterior

RAMP, receptor activity modifying protein

RCP, receptor component protein

RNA, ribonucleic acid

RT-PCR, reverse transcription polymerase chain reaction

rVLM, rostral VLM

SCh, suprachiasmatic nucleus

SFO, subfornical organ

SON, hypothalamic supraoptic nucleus

Sp5, spinal trigeminal tract

SSC, standard saline citrate

Tu, olfactory tubercle

VDB, vertical limb of the diagonal band of Broca

VLM, ventrolateral medulla

VMH, ventromedial nucleus of the hypothalamus

VP, ventral pallidum

VTg, ventral tegmental nucleus

XII, hypoglossal nucleus

LIST OF SYMBOLS

* , denotes significant difference $p < 0.05$

**, denotes significant difference $p < 0.005$

↑, denotes infusion start time

CHAPTER 1

INTRODUCTION

1.1 Overview

Adrenomedullin (ADM) is a 52 amino acid neuropeptide with both humoral and neuroendocrine effects. In the peripheral vasculature, ADM has a profound effect on blood pressure, both through its actions as a potent vasodilator, and through its diuretic and natriuretic actions (Hinson et al., 2000). In addition, ADM is produced in the central nervous system (CNS), where it acts to regulate blood pressure, feeding, salt appetite, and water balance (Hinson et al., 2000; Shan and Krukoff, 2001a). A recently characterized receptor complex for ADM is found in many tissues, including the brain. Previous reports outlining the CNS distribution of components of this ADM receptor complex are not consistent with data concerning the physiological effects of ADM in the brain (Fluhmann et al., 1997; Oliver et al., 1998; Ueda et al., 2001; Oliver et al., 2001). We have undertaken experiments aimed at resolving this conflict between the physiology of ADM and the CNS distribution of ADM signaling components. We accomplished this by first localizing messenger ribonucleic acid (mRNA) expression of ADM signaling components, then analyzing changes in levels of mRNA expression in response to changes in blood pressure.

1.2 Synthesis and Secretion of ADM

Adrenomedullin is synthesized from a 185 amino acid prepro-form, preproadrenomedullin (ppADM). Another vasoactive peptide, proadrenomedullin N-terminal 20 peptide (PAMP), is also derived from ppADM (Kitamura et al., 1993b). Although both peptides have similar biological activities, they can be differentially regulated (Uemura et al., 2002). PAMP has been suggested to act through different

central mechanisms from ADM to influence blood pressure (Samson et al., 1998), and to act on a bombesin receptor to elevate blood glucose (Ohinata et al., 2001). Figure 1.1 illustrates the processing of ADM and PAMP from the ppADM gene. ADM contains an amidated tyrosine at the carboxy terminus, and a disulfide bridge between residues 16 and 21 (Kitamura et al., 1993a). The disulfide bridge is typical of members of the calcitonin peptide family, which includes ADM, calcitonin, calcitonin gene-related peptide (CGRP), and amylin.

ADM is produced in a wide variety of tissues (Table 1.1), including the adrenal gland, heart, lung, kidney, blood vessels, and brain (Jougasaki and Burnett, Jr., 2000). In vascular smooth muscle cells, ADM is constitutively secreted (Minamino et al., 2002). Radioimmunoassays have shown that the concentration of ADM in human plasma is ~3 pmol/L (Nagata et al., 1998; Hinson et al., 2000). However, the actual concentration may be higher, as plasma radioimmunoassay may underestimate ADM concentration, due to the presence of an ADM binding protein, Factor H, in the bloodstream. Factor H may lower the available ADM in assays of plasma ADM (Pio et al., 2002). The ADM binding protein can enhance ADM function, as observed in fibroblasts, where the cyclic adenosine 3',5'-monophosphate (cAMP) stimulation by ADM is enhanced in the presence of Factor H (Pio et al., 2001b).

The synthesis and secretion of ADM is regulated by numerous physiological factors. For example, it has been reported that cortisol, aldosterone, retinoic acid, and thyroid hormone stimulate ADM production (Hinson et al., 2000). Cytokines can increase secretion of ADM, both through nitric oxide (NO) activation, and through an NO independent mechanism (Hofbauer et al., 2002). Shear stress has been shown to increase

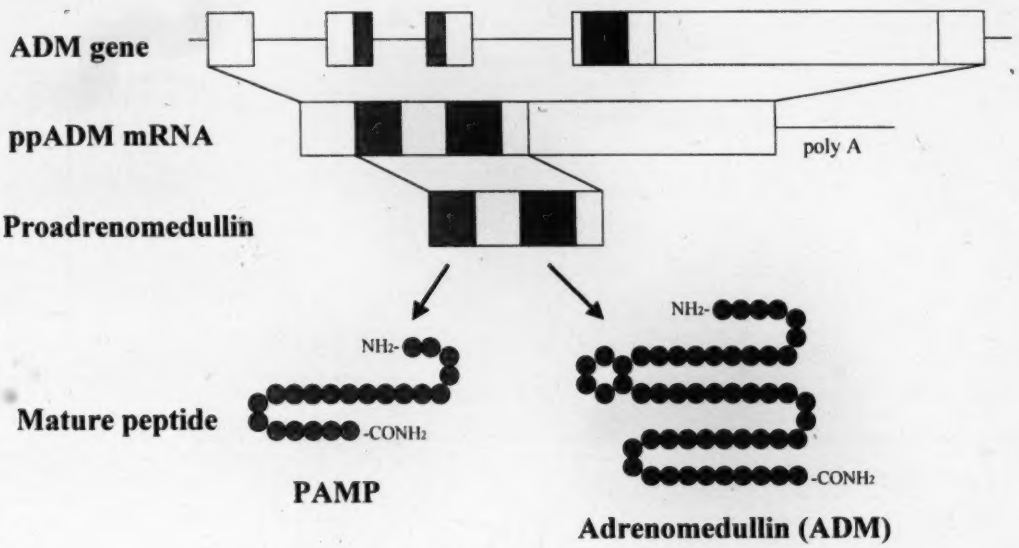


Figure 1.1: Processing of ADM and PAMP from the ppADM gene. The peptides are initially synthesized as ppADM, before enzymatic cleavage produces the mature forms of ADM and PAMP. [Modified from Shan J (2000)]

Table 1.1: Concentrations of ADM in rat tissues

Region	ADM (fmol/mg wet tissue)
Brain	0.11 ± 0.01
Cerebellum	0.08 ± 0.01
Pituitary gland	1.12 ± 0.25
Submandibular gland	0.58 ± 0.1
Thyroid gland	0.96 ± 0.12
Cardiac atrium	9.21 ± 1.48
Cardiac ventricle	0.29 ± 0.05
Aorta	0.12 ± 0.03
Lung	13.5 ± 2.8
Adrenal gland	12.2 ± 0.9
Kidney	0.32 ± 0.05
Spleen	0.22 ± 0.02
Stomach	0.51 ± 0.08
Duodenum	0.25 ± 0.02
Jejunum	0.2 ± 0.02
Ileum	0.25 ± 0.03
Cecum	0.52 ± 0.12
Colon	0.45 ± 0.05
Pancreas	0.14 ± 0.02
Liver	0.14 ± 0.01
Testis	0.15 ± 0.03
Muscle	0.07 ± 0.01
Plasma	3.60 ± 0.34

Data are mean ± S.D. (Sakata et al., 1994; Sakata et al., 1998)

(Chun et al., 1997; Dschietzig et al., 2001), or decrease (Shinoki et al., 1998) secretion of ADM. Lipopolysaccharide (LPS), a component of gram negative bacterial cell walls, can stimulate ADM production, even in the absence of cardiovascular changes (Yang et al., 2001).

1.3 ADM Receptors

ADM belongs to a family of related peptides, including calcitonin, CGRP, and amylin (Hinson et al., 2000). Recent experiments have shown that both ADM and CGRP act on the same receptor system, composed of the metabotropic calcitonin-receptor-like receptor (CRLR), and additional subunits, called receptor-activity-modifying proteins (RAMPs) that convey ligand specificity (McLatchie et al., 1998; Christopoulos et al., 1999; Sexton et al., 2001). When RAMP-2 or 3 is combined with CRLR, a specific, high affinity ADM receptor is formed (Fig. 1.2), while RAMP-1/CRLR forms a $CGRP_1$ receptor (McLatchie et al., 1998; Miret et al., 2001). The expression of RAMPs aids in trafficking CRLR to the cell surface, but may not be strictly necessary for cell surface expression (McLatchie et al., 1998; Fraser et al., 1999; Flahaut et al., 2002). An additional component of the ADM receptor is the receptor component protein (RCP), which co-precipitates with CRLR/RAMP-2, and is necessary for ADM signal transduction (Prado et al., 2001).

Several alternative receptors have been proposed as ADM receptors (Table 1.2), including the L1 (Kapas et al., 1995), RDC-1, and $CGRP_1$ receptors (Hinson et al., 2000). However, more recent experiments have failed to confirm the role of L1 as an ADM receptor (Kennedy et al., 1998). The RDC-1 and $CGRP_1$ receptors are primarily

Table 1.2: ADM receptor affinities

Proposed Receptor	Affinity: EC_{50} (nM)	Reference
RAMP-2/CRLR	4.7 ± 0.5	(Fraser et al., 1999)
RAMP-3/CRLR	4.1 ± 0.8	(Fraser et al., 1999)
$CGRP_1$ (RAMP-1/CRLR)	53.5 ± 7.9	(Fraser et al., 1999)
L1	7	(Kapas et al., 1995)
RDC-1	100	(Kapas and Clark, 1995)

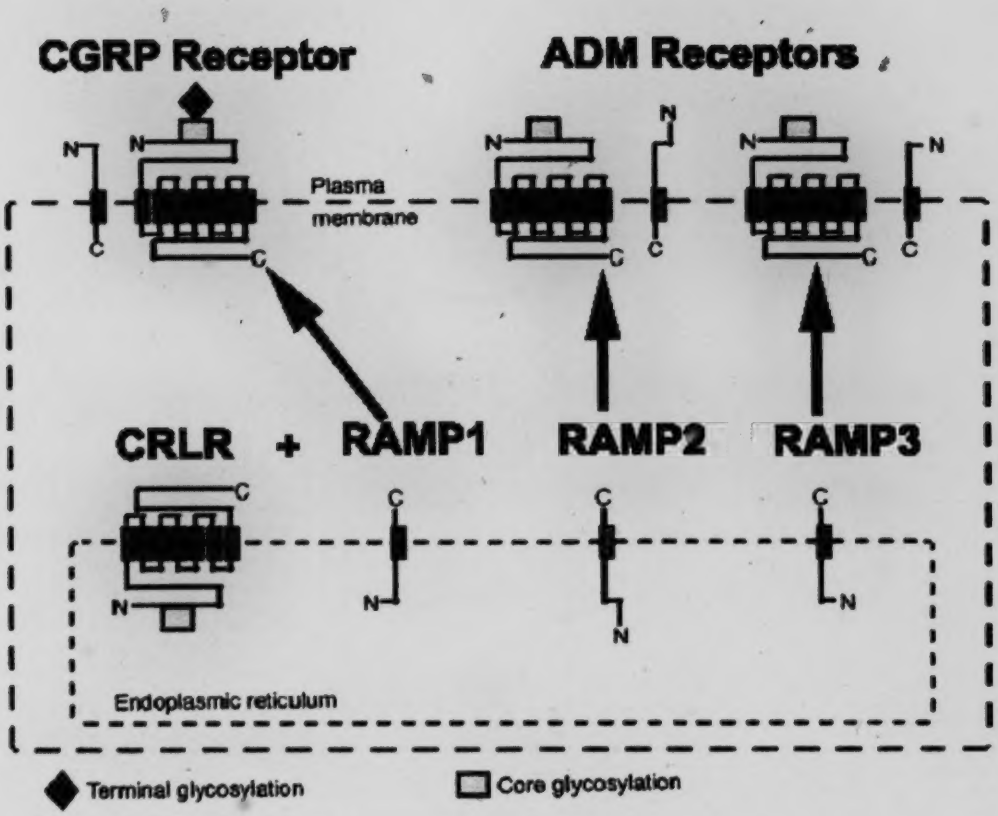


Figure 1.2: RAMPs combine with CRLR to form functional receptors. Co-expression of CRLR protein with RAMP-1 protein forms a terminally glycosylated CGRP receptor. Co-expression of CRLR protein with either RAMP-2 or RAMP-3 protein produces core glycosylated, pharmacologically similar ADM receptors. [modified from McLatchie et al. (1998)]

CGRP receptors that also bind and respond to ADM with lower affinity (Hinson et al., 2000). Since both ADM and CGRP act on the $CGRP_1$ receptor, it is not surprising that there is some cross talk between these two signaling peptides. For example, $CGRP_{8-37}$, has been used as an antagonist for both CGRP and ADM (Hinson et al., 2000).

ADM signal transduction is most often mediated by cAMP elevation via adenylyl cyclase, as is typical of the calcitonin family (Hinson et al., 2000). Additionally, other second messengers such as the phospholipase C / inositol triphosphate pathway, protein kinase C (PKC), and the mitogen-activated protein kinase (MAPK) pathway have been implicated in ADM signaling. An elevation of intracellular calcium has been observed after ADM stimulation, and may be the initial signal to stimulate NO release, as ADM can activate NO synthase (Shimekake et al., 1995; Szokodi et al., 1998). ADM stimulates the MAPK pathway in vascular smooth muscle cells, to promote cell growth (Iwasaki et al., 1998). Conversely, ADM can also inhibit MAPK production through a cAMP-dependant mechanism (Pio et al., 2001a).

Deactivation of ADM may occur through ligand triggered, clathrin-mediated endocytosis, and lysosomal degradation of the ligand/receptor complex (Kuwasako et al., 2000). It has been suggested that the RAMP/CRLR ADM receptor in the lung may act to clear ADM from the bloodstream (Dschietzig et al., 2002). Alternatively, ADM may be degraded by endogenous proteinases (Hinson et al., 2000).

1.4 The Autonomic Nervous System Regulates Blood Pressure

The autonomic nervous system (ANS) regulates blood pressure, in part, by regulating sympathetic drive. The ANS involves various, interconnected nuclei in the

CNS, which both monitor blood pressure changes and respond to them. Figure 1.3 illustrates the baroreceptor reflex, a fast-acting regulator of blood pressure that integrates information from the baroreceptors and mediates a homeostatic response. The basic baroreflex circuitry involves a pathway that projects initially from the baroreceptors to the nucleus of the solitary tract (NTS), a viscerosensory and integration center. From the NTS, the pathway proceeds to the caudal ventrolateral medulla (cVLM), then the rostral VLM (rVLM), and finally the intermediolateral cell column (IML) in the spinal cord. Each of these nuclei receives modulating influences from higher autonomic centers, such as the PVN.

An increase in blood pressure results in stimulation of the baroreceptors in the carotid sinus and aortic arch, which in turn leads to excitation of the NTS via the aortic depressor and carotid sinus nerves. The NTS, in addition to its role as a relay center in the baroreflex, has numerous functions related to viscerosensation. The rostral NTS receives gustatory information from the VII, IX, and X cranial nerves, while the caudal NTS receives viscerosensory information from the IX and X cranial nerves (Benarroch, 1997). The medial NTS receives gastrointestinal, pulmonary, respiratory, and cardiovascular information, and plays a major role in the baroreceptor reflex (Benarroch, 1997). The NTS is extensively interconnected with other central autonomic nuclei. For example, the NTS is closely associated with the area postrema (AP). In fact, some NTS neurons send dendrites into the AP (Saper, 1995). This association allows humoral signals to be transmitted to the NTS (see section 1.6).

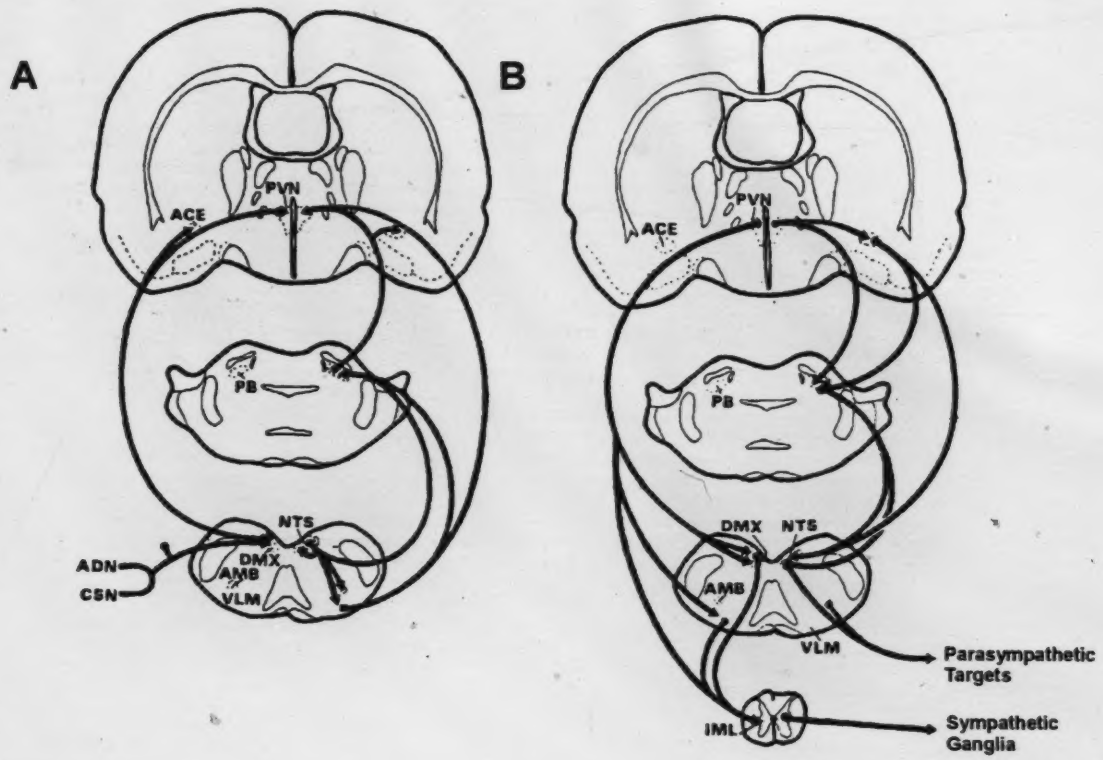


Figure 1.3: The afferent (A) and efferent (B) limbs of the baroreflex. The baroreflex consists of the aortic depressor and carotid sinus nerves, which carry information from the baroreceptors to the NTS. From there, information is transmitted to integration and processing centers in the PVN, PB, ACE, DMX, and VLM. These nuclei regulate sympathetic output via direct and indirect projections to the IML, which innervates sympathetic ganglia. [modified from Calaresu et al. (1984)]

As illustrated in Figure 1.3A, the NTS sends projections to numerous autonomic nuclei, including the hypothalamic paraventricular nucleus (PVN), the central amygdaloid nucleus (ACE), the parabrachial nucleus (PB), the dorsal motor nucleus of the vagus (DMX), the nucleus ambiguus (AMB), and the VLM. Figure 1.3B illustrates how many of these projections are reciprocal. For example, the NTS has reciprocal catecholaminergic projections throughout the rostral-caudal extent of the VLM, and these neurons are functionally activated by ACE stimulation (Petrov et al., 1996). The output from the NTS to the VLM regulates sympathetic outflow during baroreceptor reflex activation. NTS stimulation results in stimulation of the caudal VLM (cVLM). The cVLM then inhibits the rostral VLM (rVLM), an area that produces tonic sympathetic outflow to the sympathetic preganglionic neurons in the IML (Sved et al., 2001). Therefore, an increase in blood pressure results in an inhibition of the rVLM, and a decrease sympathetic drive. This decrease restores blood pressure by reducing heart rate and peripheral resistance.

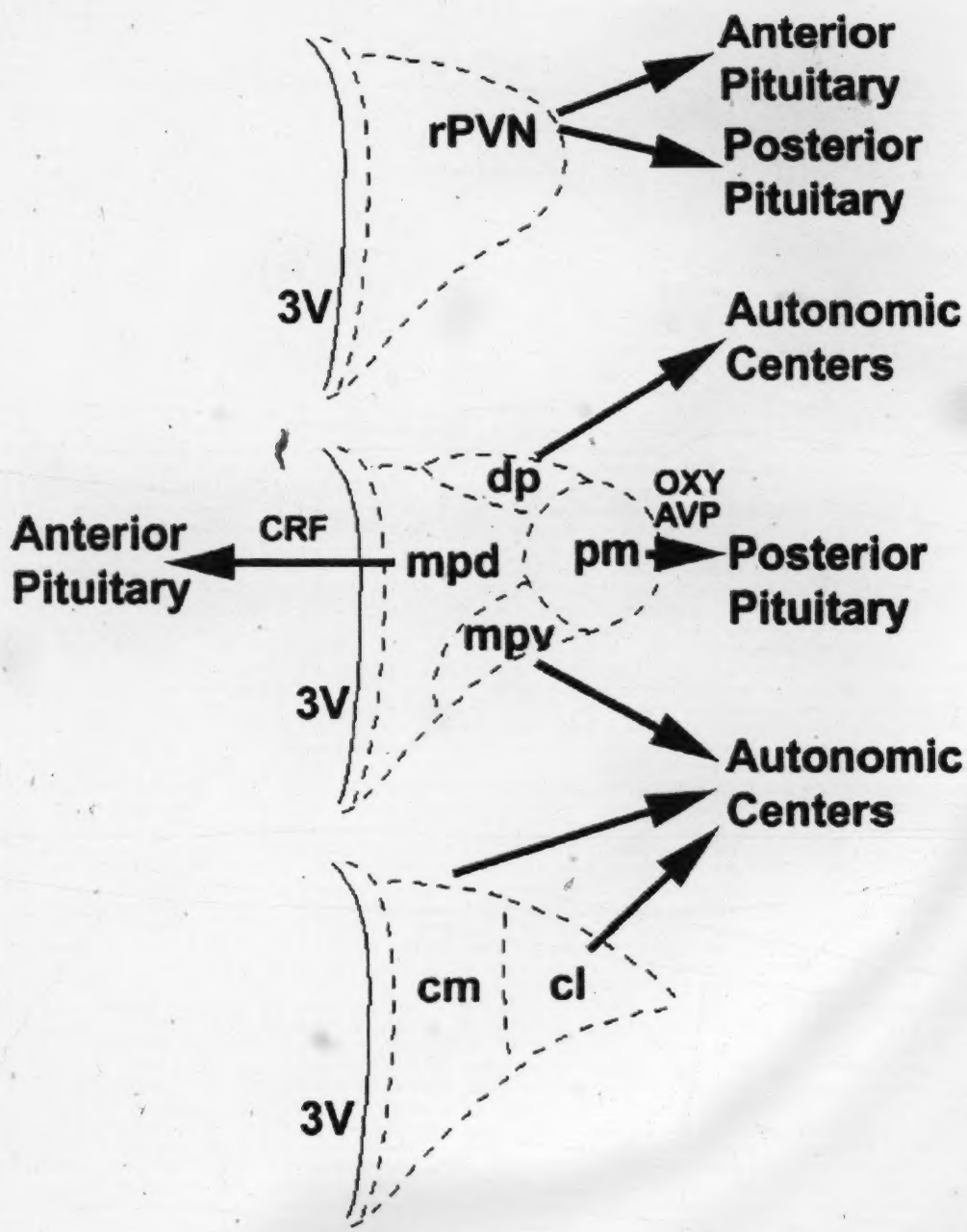
The basic baroreceptor circuitry is modulated by reciprocal interconnections with higher centers in the central autonomic system, such as the PVN. The PVN coordinates information about blood pressure with information about physiological state, for example, fluid and electrolyte balance or food intake (Sewards and Sewards, 2000; Schwartz et al., 2000). Like the NTS, the PVN alters sympathetic outflow via neurons that project to the cVLM (Pyner and Coote, 2000). In addition, the PVN regulates endocrine function by regulating the activity of the anterior pituitary.

The multiple roles of the PVN are carried out by functionally distinct subdivisions (Fig. 1.4). Neurons of the posterior magnocellular subdivision (pmPVN) project to the

posterior pituitary (PP), to regulate oxytocin (OXY) and arginine-vasopressin (AVP) release into the bloodstream. AVP is a pressor agent, acting through peripheral vasoconstriction and in the kidney to retain water and salt (Bennett and Gardiner, 1985; Baylis, 1987). OXY has a variety of roles relating to sexual function and coping with stress (Uvnas-Moberg, 1997). The parvocellular PVN is further subdivided into discrete functional groups. The dorsolateral medial parvocellular subdivision (mpdPVN) projects to the median eminence, to regulate release of anterior pituitary hormones. For example, both corticotropin releasing factor (CRF) and AVP are produced in the parvocellular PVN (pPVN), and can stimulate adrenocorticotropic hormone (ACTH) release from the anterior pituitary (Clarke, 1996).

Numerous studies of PVN projections to other autonomic nuclei have been performed, using both anterograde and retrograde tracers. The dorsal parvocellular (dpPVN) and ventral medial parvocellular (mpvPVN) subdivisions as well as the caudal lateral (clPVN) and caudal medial PVN (cmPVN) subdivisions of the PVN project to autonomic nuclei to regulate sympathetic output (Sawchenko and Swanson, 1982). Targets of these subdivisions include autonomic nuclei such as the NTS and VLM, and the IML nucleus of the spinal cord (Petrov et al., 1995; Pyner and Coote, 2000; Hallbeck et al., 2001). The neurons of these subdivisions have been shown to contain numerous neurochemicals, including catecholamines, dynorphin, enkephalin, oxytocin, and AVP (Petrov et al., 1995; Krukoff et al., 1997).

Figure 1.4: PVN subdivisions have specific functions (Armstrong, 1995). The rPVN has projections to both the anterior and posterior pituitary. The pm subdivision regulates AVP and OXY release from the posterior pituitary. The mpd subdivision produces releasing factors such as CRF to regulate anterior pituitary function and the HPA axis. The dp, mpv, cm, and cl subdivisions project to autonomic nuclei to regulate sympathetic drive. For abbreviations, see list.


Anterior
Pituitary
rPVN
Posterior
Pituitary
3V
Autonomic
Centers
dp
OXY
AVP
Anterior
Pituitary
CRF
mpd
pm
Posterior
Pituitary
mpv
3V
Autonomic
Centers
cm
cl
3V

1.5 Physiology of ADM

In the peripheral vasculature, ADM acts as a potent hypotensive agent, acting via vasodilation, as well as diuresis and natriuresis to reduce blood volume (Hinson et al., 2000). The overall effect of ADM in the peripheral vasculature is to decrease blood pressure. For example, during septic shock, the elevation of ADM results in drastic decreases in blood pressure (Nishio et al., 1997). It has been suggested that the decrease in blood pressure elicited by ADM may be counterbalanced by the activity of ADM in the CNS, where it acts to increase blood pressure (Hinson et al., 2000; Shan and Krukoff, 2000). This hypothesis is supported by the observation that peripheral ADM can signal the CNS via circumventricular organs (Shan and Krukoff, 2000). ADM also produces positive ionotropism and chronotropism of the heart, likely due to calcium elevation and PKC activation (Parkes and May, 1997; Szokodi et al., 1998). In addition, ADM can inhibit food intake and gastric emptying (Reidelberger et al., 2002).

Many disease states are associated with increased plasma ADM, including hypertension, septic shock, and hypoxia. Stimuli which elicit ADM secretion under these conditions include shear stress or pressure overload on the vasculature in hypertension, oxidative stress and free radical production during hypoxia, and the presence of LPS in septic shock (Yoshihara et al., 2000; Yang et al., 2001; Yoshihara et al., 2002). It is not always clear whether release of ADM is a cause or consequence of these conditions.

In many cases, ADM has been shown to prevent tissue damage in pathological conditions (Dobrzynski et al., 2000; Chao et al., 2001; Nishimatsu et al., 2002; Nishikimi et al., 2002). The involvement of ADM in the pathophysiology of hypertension has been investigated in many human studies, and also in a wide variety of rat models of

hypertension. The increase in plasma ADM associated with these conditions is related to the increase in blood pressure. For example, plasma ADM was elevated in patients with pulmonary hypertension, and ADM levels were correlated to the mean arterial pressure (MAP) (Kakishita et al., 1999). Patients with atherosclerosis showed increases in plasma ADM associated with both the elevation of MAP and the severity of atherosclerosis (Shinomiya et al., 2001). ADM plasma levels are also increased in pregnancy, a condition associated with increased blood pressure (Nagata et al., 1998). Animal experiments have indicated that in hypertension, ADM likely has a protective role. Infusion of ADM improved survival in rats with malignant hypertension (Mori et al., 2002). ADM knockout mice showed underdeveloped vasculature and died *in utero*, while heterozygotes showed elevated blood pressure and reduced NO production (Shindo et al., 2001). When administered angiotensin II and salt, heterozygote mice showed vascular damage, and signs of oxidative stress (Shimosawa et al., 2002). ADM transgenic mice over-expressing the ADM gene showed reduced kidney and heart damage in several rat models of hypertension (Chao et al., 2001). ADM gene therapy reduced renal injury and cardiac hypertrophy in hypertensive rats (Dobrzynski et al., 2000). ADM gene delivery significantly reduced blood pressure, renal injury, and cardiac remodeling in hypertensive rats, as well as cardiac injury in myocardial ischemic rats (Dobrzynski et al., 2000; Chao et al., 2001).

ADM is also found to be renal protective in hypertension. ADM reduced renal injury in Dahl salt-sensitive hypertensive rats, without altering blood pressure (Nishikimi et al., 2002). Additionally, heart failure transiently increased renal ADM (Yoshihara et al., 2001). In response to renal ischemia, heterozygote ADM knockout mice showed

significantly more renal damage than controls, while ADM transgenic mice showed significantly less damage than controls (Nishimatsu et al., 2002).

Hypoxia also increases ADM protein levels (Cuttitta et al., 2002). Following cerebral ischemia, ADM protein levels were increased, and found to be neuroprotective (Serrano et al., 2002). ADM mRNA levels were increased in cerebral microvasculature cells and in cultured neuroblastoma cells in response to hypoxia (Ladoux and Frelin, 2000; Kitamuro et al., 2001). During hypoxia, ADM and RAMP-2 may be differentially regulated, as hypoxia caused a decrease of RAMP-2 mRNA levels in neuroblastoma cells (Qing et al., 2001; Kitamuro et al., 2001).

1.6 Circulating ADM signals the brain

ADM is thought to act as an endocrine factor in the peripheral vasculature. The potent effects of ADM on the vasculature to produce vasodilation suggest that circulating ADM can act as a hormone. The significance of ADM as a hormone has been questioned, however, because the pM concentrations of circulating ADM are considered insufficient to activate receptors which bind ADM with nM affinity (Hinson et al., 2000; Pio et al., 2002). As discussed above, the ADM binding protein may result in underestimation of circulating ADM levels, and can also increase ADM function (Pio et al., 2001b). The existence of the binding protein therefore adds credence to the suggestion that ADM acts as a hormone.

Many hormones are known to signal the brain via circumventricular organs (CVOs), specialized vascular structures that lack a blood-brain barrier, and therefore permit access of substances from the periphery to the CNS (Oldfield and McKinley,

1995). A recent study has shown that ADM signals the CNS via the AP, a CVO (Shan and Krukoff, 2000). Using Fos expression as an indicator of cell activation, it was shown that ADM signals the NTS through the adjacent AP. The effects of ADM may be mediated by activation of AP neurons (Allen and Ferguson, 1996), or possibly by direct actions on NTS dendrites, which extend into the AP (Saper, 1995). Further, intravenous (IV) ADM activated PVN neurons, and this activation was attenuated by AP ablation (Shan and Krukoff, 2000). Activation of these neurons is interesting in view of the fact that AP, NTS, and PVN neurons express ppADM (Shan and Krukoff, 2001a). These observations suggest that peripheral ADM can signal through CVOs to activate the central ADM system. However, as a detailed description of ADM receptors in the CNS is lacking, this hypothesis is incomplete.

1.7 Central Actions of Adrenomedullin

ADM is produced in the CNS, where it acts to regulate blood pressure, feeding, and salt appetite (Hinson et al., 2000). ADM produced in the brain also has a physiological role in water drinking, as disruption of endogenous ADM production with ribozyme injection resulted in exaggerated drinking (Taylor and Samson, 2002). Consistent with these observed effects, ppADM mRNA was found in a number of autonomic nuclei, such as the PVN, NTS, ACE, ventromedial hypothalamus (VMH), arcuate nucleus (Arc), and supraoptic nucleus (SON) (Shan and Krukoff, 2001a). Notably, intracerebroventricular (ICV) injection of ADM activated these same nuclei, as measured by expression of c-fos (Ueta et al., 2001; Shan and Krukoff, 2001b). However,

since c-fos is also activated by changes in blood pressure, it is not clear whether ADM can act directly on these nuclei, or acts indirectly through its effects on blood pressure.

ADM can both stimulate and inhibit neurons, as demonstrated by alterations in current of diagonal band of broca (DBB) neurons in response to ADM (Shan et al., 2002), and by in vitro recordings from AP neurons (Allen and Ferguson, 1996). Therefore, the actions of ADM in the CNS to regulate blood pressure are heterogeneous. ADM increases blood pressure by increasing sympathetic drive when injected into the cerebral ventricles or microinjected into the AP, decreases blood pressure when microinjected into the PVN, and has no effect on blood pressure when microinjected to the NTS (Allen et al., 1997; Samson et al., 1998; Smith and Ferguson, 2001).

Central ADM also has neuroendocrine effects, inhibiting stimulated AVP release (Yokoi et al., 1996; Serino et al., 1999), while stimulating OXY and ACTH release (Charles et al., 1997; Shan and Krukoff, 2001b). AVP acts both centrally and peripherally to elevate blood pressure, by influencing autonomic drive and promoting water and salt retention in the kidney (Bennett and Gardiner, 1985). Inhibition of AVP is consistent with the role of ADM in the periphery to decrease blood pressure, and stimulate diuresis and natriuresis (Hinson et al., 2000). The stimulation of ACTH secretion by ICV ADM indicates that ADM stimulates the hypothalamo-pituitary-adrenal (HPA) axis, a reflex pathway active when an animal is under stress. It has been suggested that stimulation of the HPA axis may occur at the level of the PVN (Shan and Krukoff, 2001b), but specific ADM receptors have not yet been shown in the PVN.

1.8 ADM Receptor Expression in the CNS

A significant correlation exists between RAMP-2 and CRLR mRNAs in a wide variety of tissues, including heart, lung, liver, spleen, spinal cord, cerebellum, and vas deferens (Chakravarty et al., 2000). However, reports of the CNS distribution of RAMP-2 and CRLR mRNA showed quite different expression patterns. RAMP-2 mRNA was found in the olfactory bulb, diagonal band of Broca, hippocampus, amygdala, dorsomedial, ventromedial, arcuate, and anterior hypothalamic nuclei, the nucleus of the solitary tract, facial nucleus, and cerebellum (Ueda et al., 2001; Oliver et al., 2001). CRLR mRNA appeared to be more focally expressed, in the caudal caudate putamen, amygdala, the piriform cortex, and amygdalostriatal transition area (Fluhmann et al., 1997; Oliver et al., 1998). The only area described so far as expressing both RAMP-2 and CRLR is the amygdaloid region. Autoradiographic binding studies of labeled ADM showed binding in this region, suggesting that a high concentration of ADM receptors is present (Juaneda et al., 2001).

Previous reports of the expression patterns of ADM receptors are difficult to reconcile with the reported actions of ADM in the CNS. ADM has been shown to alter blood pressure when it is microinjected into the PVN or the AP, and has also been shown to activate cells in the NTS by way of the AP (Allen et al., 1997; Shan and Krukoff, 2000; Smith and Ferguson, 2001). These nuclei should therefore contain an ADM receptor. Additionally, ADM is produced in reciprocally interconnected autonomic nuclei, which are activated by ICV ADM (Calaresu et al., 1984; Ueta et al., 2001; Shan and Krukoff, 2001a; Shan and Krukoff, 2001b). An ADM receptor should therefore be present in these same nuclei, to permit ADM signal transduction. These data suggest that

a specific ADM receptor should be located in the PVN, the NTS, the AP, and in other autonomic nuclei such as the SON and VLM. In this thesis, we will describe experiments demonstrating that RAMP-2 and CRLR are present in these nuclei.

1.9 Hypothesis

We propose that (A) **RAMP-2 and CRLR are present in central autonomic nuclei,** and (B) **receptor expression is regulated by changes in blood pressure.** We will describe the CNS expression of an ADM receptor, using RAMP-2 and CRLR mRNA expression as an indicator of receptor expression. We will also show changes in both RAMP-2 and ppADM mRNA in autonomic nuclei in response to alterations in blood pressure.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Male Sprague-Dawley rats (230-560 g) were purchased from the Biological Sciences Animal Center at the University of Alberta. They were housed in the Health Sciences Lab Animal Services (HSLAS) conventional housing, on a 12h/12h light/dark cycle, with free access to food and water. The University of Alberta Animal Welfare Committee approved all protocols.

2.2 Experimental Design

2.2.1 RAMP-2 mRNA Survey

Two control animals from the experiments outlined in section 2.2.3 were used to examine the CNS distribution of RAMP-2. These rat showed no changes in MAP in response to vehicle infusions. Rats were deeply anesthetized with sodium pentobarbital, and perfused transcardially with 200 ml physiological saline, followed by ice cold 4% paraformaldehyde (Sigma Chemical Co, St. Louis, MO; % indicates w/v) in phosphate buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, 0.02% KH_2PO_4). Brains were removed and post-fixed in 2% paraformaldehyde/15% sucrose for one hour, before overnight storage in 20% sucrose. Coronal brain sections were cut at a thickness of 25 μm in a cryostat. Sections were thaw-mounted in pairs, with sections from experimental and control animals on the same slide, dried, and stored at –70°C until use.

Tissue was processed for *in situ* hybridization (ISH) as described in section 2.3. Brain sections from control animals were examined with dark field microscopy to identify areas containing RAMP-2 signal, and with light field microscopy to confirm the

anatomical location of the signal. Sections processed with sense probe showed no positive signal in the areas examined.

2.2.2 CRLR mRNA Survey

For the ISH experiments, rats were processed as above. For the RT-PCR experiments, two rats were deeply anesthetized with Urethane (Sigma, 1.75g/kg) and decapitated. Brains, spinal cords, and lungs were removed, rinsed in 20% sucrose, and dissected. A forebrain section was removed, corresponding to approximately –0.8 mm to –3.6 mm [relative to bregma (Paxinos and Watson, 1986)]. Tissue blocks from the hypothalamus and amygdala were dissected as follows: hypothalamus, 0-2 mm lateral to the midline, 7-10 mm ventral to the dura; amygdala, 3-7 mm lateral to the midline, and 7-10 mm ventral to the dura. A tissue block of the brainstem corresponding to –13.5 mm to –14.0 mm (from bregma) and 0-2.5 mm lateral to the midline was removed. Both left and right tissue blocks were included. The cerebellum was cut along the midline, and only half was used. The C2-C3 region of the spinal cord was also removed. These tissue blocks (50-100 mg), along with 100 mg lung tissue, were homogenized to extract the RNA. The RNA was used for RT-PCR analysis, described in 2.3.5.

2.2.3 Modulation of RAMP-2 and ppADM mRNA expression

2.2.3.1 Surgical Procedures

Rats were instrumented with arterial and venous catheters as previously described (Krukoff et al., 1997; Yang et al., 1999). In the HSLAS surgical suites, rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.; Somnotol, M.T.C.

Pharmaceuticals, Cambridge, ON), and received buprenorphine hydrochloride (16 μg i.m.; Buprenex® Injectable, Reckitt & Colman Pharmaceuticals Inc, Richmond, VA), and Duratears® ophthalmic ointment (Alcon Canada Inc, Mississauga, ON). A midline incision was made, and the descending aorta and inferior vena cava were exposed and cannulated (Intramedic™ PE10 tubing I.D. 0.28mm, O.D. 0.61mm, Becton Dickinson and Co, Sparks, MD; Silastic® tubing I.D. 0.51mm, O.D. 0.94mm, Dow Corning Corp, Midland, MI). The lines were tunneled under the skin from abdomen to back, and externalized at the nape of the neck with stainless steel tubing (27-gauge and 23-gauge, Small Parts Inc, Miami Lake, FL), and the arterial line was sealed with polyvinylpyrrolidone (0.8 g/mL PVP-40, Sigma). After a recovery period of 4-5 days, the arterial line was connected to a pressure transducer to monitor blood pressure in conscious, freely moving rats, and the venous line was connected to an infusion pump.

2.2.3.2 Effect of Nitroprusside on RAMP-2 and ppADM mRNA levels

Following a 30-minute baseline pressure measurement, mean arterial pressure (MAP) was lowered by IV infusion of nitroprusside [NP, Sigma, 2.5 mg/ml (Jhamandas et al., 1998)]. Control rats received equivalent volumes of physiological saline (vehicle). The reduced MAP was maintained for 6 hours in the NP rat ($p<0.001$). The IV infusion rate was adjusted over the 6-hour period to maintain the MAP at about 20% lower than baseline (average rate ≈ 5μL/min).

2.2.3.3 Effect of Phenylephrine on RAMP-2 and ppADM mRNA levels

Following a 30-minute baseline pressure measurement, MAP was elevated by IV infusion of phenylephrine (PHENYL, Sigma, 2 mg/ml), again paired with vehicle treated

rats. The elevated MAP was maintained for 6 hours in the PHENYL rat ($p<0.001$). As before, the IV infusion rate was adjusted over the 6-hour period to maintain the MAP at about 20% above baseline (average rate ≈ 5.5μL/min). Immediately following the experiment, rats were perfused as described above in 2.2.1

2.3 Molecular Protocols

2.3.1 *In situ* hybridization (ISH) Probe Preparation

Total rat lung RNA was extracted using Trizol reagent (Invitrogen Canada, Burlington, ON), and reverse transcription polymerase chain reaction (RT-PCR) produced a 335 base pair RAMP-2 complementary deoxyribonucleic acid (cDNA). The RT reaction mix consisted of: 5μl RNA, 7μl 2.5 mM dNTP, 2μl PCR buffer, 1μl random hexamers, 1μl RNAse inhibitor, 1μl M-MLV, 2μl 100mM dithiothreitol (DTT), and 1μl 50 mM $MgCl_2$. The reaction mix was incubated at 20° for 10 minutes, 42° for 1 hour, and 95° for 5 minutes. The PCR reaction mix consisted of: 20μl DNA, 8μl PCR buffer, 20μl 2.5 mM dNTP, 4μl sense primer TGGATCACAGCTCGCTGTGA, 4μl antisense primer CCAAGGGATTTGGGAAGCCC, 0.5μl Taq, 10μl DTT, 6μl 50 mM $MgCl_2$, and 27.5μl H_2O. The PCR mix was incubated at 94° for 45 seconds, 55° for 1 minute, and 72° for 1 minute for 30 cycles, followed by 72° for 5 minutes. Sequencing the cDNA confirmed the fragment to be nucleotides 50-384 of the RAMP-2 mRNA (Genbank Accession AF181551).

The cDNA was then ligated into pGEM-T vector (Promega, Madison, WI) by incubating 5 ng cDNA with 50 ng pGEM-T, 1 μl T4 DNA ligase, and 5 μl 2X Rapid ligation buffer overnight at 4°C. The resulting product was then transformed into DH5α

bacteria. The cells were plated on LB plates with ampicillin/IPTG/X-Gal to select for transformants. These cells were grown to amplify the plasmid, and a maxi-prep (Promega) produced purified plasmid suitable for probe synthesis. Antisense ^{35}S-UTP labeled probes were generated by linearizing the plasmid with *Sac*I, and transcribing with T7 polymerase. Sense probes were generated with *Apa*I/SP6 (enzymes from Promega).

The ppADM cDNA was kindly donated by Dr. E.J. Taparowsky (Purdue University). Antisense ^{35}S-UTP labeled probes were generated with *Bam*HI/T7. Sense probes were generated with *Apa*I/T3 (enzymes from Promega).

The probe synthesis reaction consisted of: 2µl ^{35}S-UTP, 2µl 2.5mM CTP/GTP/ATP, 2µl TSC Buffer, 2µl 0.5 µg/µl template, 0.7µl 100mM DTT, 0.3µl RNAse Inhibitor, and 1µl polymerase, incubated for 1.5 hours at 37°C. The template DNA was then digested with RQ1 DNAse. The probe was purified with Biospin® 30 chromatography columns (Bio-Rad Laboratories, Hercules, CA), precipitated with 66% ethanol/0.75M ammonium acetate, and resuspended in SET (20% sodium dodecyl sulphate, 10mM Tris-HCl, pH 7.5, 1mM EDTA, 10mM DTT). DTT (final concentration: 100mM), and 0.5 mg yeast transfer RNA were added, and the probe was diluted in hybridization buffer (50% formamide, 10% dextran, 0.1M NaCl, 1x Denhardt's solution, 10 mM Tris-HCl, pH 8, 1mM EDTA) to a concentration of 3 million cpm/80 µl.

A 272 base pair cDNA representing an intracellular domain of CRLR (nucleotides 1572-1843, Genbank accession # NM_012717) was kindly donated by Dr. David Poyner (Aston University, UK). The cDNA was ligated into pGEM-T vector, and sequenced to confirm its identity. Antisense probes were generated with *Nco*I/SP6, and sense probes were generated with *Pst*I/T7. ^{35}S labeled probes were generated as described above.

2.3.2 Hybridization

^{35}S *in situ* hybridization was performed to quantitate the mRNA expression of RAMP-2, CRLR and ppADM. The following *in situ* hybridization protocols have been previously described (Krukoff et al., 1999; Yang and Krukoff, 2000). Slides were warmed to room temperature, pre-hybridized with 4% paraformaldehyde, phosphate buffered saline, (PBSx2), 20 μg/ml proteinase K (Invitrogen) in 50 mM Tris/HCl (pH 8) and 10mM EDTA, PBS, 0.25% acetic anhydride in 1 mM triethanolamine (pH 8), and dehydrated in 70 and 80% ethanol/sodium acetate, then 95% ethanol (x2).

Radiolabeled probes were applied at a concentration of 3 million cpm/slide, in a volume of 80 μL. Slides were coverslipped, and incubated in humidified chambers at 45°C overnight. The coverslips were removed, and the slides were washed twice in 4x standard saline citrate (SSC: 0.9% NaCl, 0.4% sodium citrate) with 2-mercaptoethanol (Sigma, 1 μl/ml), and twice in 4x SSC. Slides were then incubated with RNAse A (Sigma, 10 μg/ml) in STE buffer (10 mM Tris/HCl, pH 8, 250 mM NaCl, 1mM EDTA), for 30 min at 37°C, followed by 30 min in STE at 37°C, 40 min in 2x SSC at 42°C, and 45 min in 0.1x SSC at 65°C. Sections were air dried and placed on x-ray film (X-OMAT AR, Kodak, Rochester, NY) for 3-4 days, then dipped in 50% NTB-2 Kodak emulsion, and exposed for 14-21 days. Slides were developed for autoradiography in D-19 developer and Rapid Fixer (Kodak), stained with 0.5% cresyl violet acetate (Sigma, pH 3.9), dehydrated, and coverslipped. The cresyl violet counterstain specifically stains neurons.

2.3.3 ISH Signal Analysis

Sections were examined with dark field microscopy to quantitate signal, and with light field microscopy to locate discrete nuclei. Nuclei were subdivided as previously described (Yang et al., 1999; Shan and Krukoff, 2000; Yang and Krukoff, 2000). The PVN was divided into rostral, middle and caudal subdivisions according to Paxinos and Watson (Paxinos and Watson, 1986), as follows: rostral (from bregma) –1.55 to –1.70 mm, middle –1.70 to –1.85 mm, caudal –1.85 to –2.00 mm. The NTS was divided as follows: rostral -13.1 to –13.5 mm, middle –13.5 to –14.0 mm, caudal –14.0 to –14.6 mm. The VLM was divided as follows: rostral -13.1 to –13.5 mm, caudal –14.0 to –14.6 mm. The VLM was considered to be the area between the ventral surface of the medulla and the nucleus ambiguous, and no attempt was made to further subdivide this nucleus.

A photograph of each area of interest was taken with a digital camera (DC330, DAGE MTI Inc. Michigan, MI), and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used to analyze the image. Signal intensity was quantified as previously described (Yang and Krukoff, 2000). A boundary was drawn around the area of interest, and the percentage of area covered with silver grains was calculated. An identical boundary was drawn around a nearby region containing nonspecific background signal, and the background measurement was subtracted from each measurement for positive signal. Measurements from two or more sections per animal were averaged where possible, and each measurement was replicated in at least five animals for statistical analysis. Data are expressed as mean ± standard error.

2.3.4 Production of photomicrographs

Photomicrographs were captured under dark field illumination, using a digital camera (Axiocam MRc, Carl Zeiss Inc., Thornwood, NY), mounted on a Zeiss Axioplan 2 microscope. Adobe Photoshop v. 5.0 was used to process the images.

2.3.5 CRLR RT-PCR

Total RNA was extracted from lung, hypothalamus, brain stem, amygdala, cerebellum, and spinal cord by homogenization in Trizol reagent, and RT-PCR was performed as in 2.3.1. CRLR primers were TCATTGTGGTGGCTGTGTTT (sense) and AATGGGACCATGGATGATGT (antisense), which produced a 176 bp cDNA (n. 1397-1572, Genbank L27487). Actin primers were GTGGGGCGCCCCAGGCACCA (sense), and GTCCTTAATGTCACGCACGATTTC (antisense), which produced a 536 bp cDNA (n.103-638 Genbank NM_031144). Both CRLR and actin primers were added to the PCR mix, to generate two separate bands for semi-quantitative analysis. The RT enzyme was left out of one reaction, to control for genomic DNA contamination. The RT-PCR reaction and analysis were repeated four times to verify the results. A photograph of the ethidium bromide stained gel was scanned, and analyzed with Scion Image software (Scion Corporation, Frederick, MD). The intensity of the CRLR band was divided by the intensity of the actin band.

2.4 Statistics

MAP and heart rate (HR) were calculated using Advanced CODAS software (DATAQ Instruments, Akron OH). The MAPs of treatment groups, as well as the CRLR

RT-PCR data, were subjected to a one-way ANOVA, with Tukey's multiple comparison post-hoc test, using Prism v. 3.00 (GraphPad Software, San Diego, CA). For the ISH quantitation of mRNA, Student's t-test was used to compare values between treatment groups. A p-value of < 0.05 was considered significant.

CHAPTER 3

RESULTS

3.1 RAMP-2 mRNA Distribution

The distribution of RAMP-2 mRNA in the central nervous system is summarized in Table 3.1. Figure 3.1 shows dark field photomicrographs of various nuclei containing RAMP-2 mRNA in the CNS. Figure 3.2 illustrates the relative intensity and location of cells expressing RAMP-2 mRNA signal.

Telencephalon

Moderate to high levels of RAMP-2 mRNA were found in the cerebral cortex and hippocampus. Specifically, the CA1, CA2, and CA3 regions of the hippocampus showed high levels, while the dentate gyrus of the hippocampus, and cortical layers 2 and 3 showed moderate levels of signal. Little or no signal was seen other cortical layers. Low levels of signal were also found in the olfactory tubercle, piriform cortex, the septal nucleus, the ventral pallidum, the diagonal band of Broca, the subfornical organ, and throughout the amygdala.

Diencephalon

The hypothalamus showed high levels of signal, especially in the supraoptic and paraventricular nuclei. Low levels of RAMP-2 mRNA were also present in the preoptic, suprachiasmatic, periventricular, arcuate, and ventromedial nuclei.

The thalamus showed low levels of signal in the anterodorsal, central medial, mediodorsal, and paraventricular nuclei.

Mesencephalon/Metencephalon

Little or no signal was found in the mesencephalon. RAMP-2 mRNA was present in the Purkinje cell layer of the cerebellum, the locus coeruleus, the parabrachial nucleus, the dorsal raphe nucleus, and the dorsal tegmental nucleus.

Table 3.1: Distribution and relative levels of RAMP-2 mRNA in the rat brain (Areas according to Paxinos and Watson, 1986)

Area	Relative Abundance
Telencephalon	
Cerebral cortex	++
Tenia tecta	-
Corpus callosum	-
Olfactory tubercle	+
Piriform cortex	+
Caudate putamen	-
Accumbens nucleus	-
Bed nucleus, stria terminalis	-
Septum nucleus	
Lateral	+
Medial	+
Ventral pallidum	+
Nucleus of the diagonal band (Broca)	+
Globus pallidus	-
Substantia innnominata	-
Hippocampus	
CA1, CA2, CA3 regions of Ammon's Horn	+++
Dentate gyrus	++
Amygdala	
Basolateral nucleus	+
Central nucleus	+
Medial nucleus	+
Cortical nucleus	+
Diencephalon	
Hypothalamus	
Anterior nucleus	-
Preoptic hypothalamic area	+
Lateral hypothalamic area	-
Suprachiasmatic nucleus	+
Paraventricular nucleus	++
Periventricular nucleus	+
Supraoptic nucleus	+++
Arcuate nucleus	+
Dorsomedial nucleus	-
Ventromedial nucleus	+
Mammillary nucleus	-
Thalamus	
Anterodorsal nucleus	+
Anteroventral nucleus	-
Anteromedial nucleus	-

Central medial nucleus	+
Mediodorsal nucleus	+
Laterodorsal nucleus	-
Lateroposterior nucleus	-
Posteroventral nucleus	-
Paraventricular nucleus	+
Habenular nucleus	-
Zona incerta	-
Mesencephalon	-
Metencephalon	
Cerebellum	
Purkinje cell layer	+
Granule cell layer	-
Cerebellar deep nuclei	-
Locus coeruleus	+
Parabrachial nucleus	
Lateral	+
Medial	+
Dorsal raphe nucleus	+
Abducens nucleus	-
Motor trigeminal nucleus	-
Dorsal tegmental nucleus	+
Myelencephalon	
Raphe obscurus nucleus	-
Facial nucleus	-
Paragigantocellular reticular nucleus	++
Ambiguus nucleus	-
Hypoglossal nucleus	+++
Nucleus of the solitary tract	+
Dorsal motor nucleus of the vagus	+
Ventrolateral medulla	++
Interstitial nucleus	-
Inferior olivary nucleus	+
Spinal trigeminal nucleus	+
Gracile nucleus	+
Cuneate nucleus	+
Vestibular nucleus	-
Circumventricular organs	
Area postrema	+
Subfornical organ	+
Pituitary	
Posterior lobe	+
Anterior lobe	-
Intermediate lobe	-

+++ strong signal, ++ moderate signal, + weak signal, - no detectable signal

Figure 3.1: Photographs of RAMP-2 *in situ* hybridization in areas of the CNS. Relative intensities, as listed in Table 3.1, are indicated for each dark field photomicrograph. (A) The cerebral cortex (++). (B) The hippocampus (+++). (C) Brainstem areas, including the AP (+), NTS (+), DMX (++), and hypoglossal nucleus (+++). (D) The supraoptic nucleus (+++). (E) The hypothalamic PVN (++). (F) The sense control probe (-) shows no RAMP-2 signal in the PVN or other nuclei. For abbreviations, see list.

Calibration bars = 100μm.

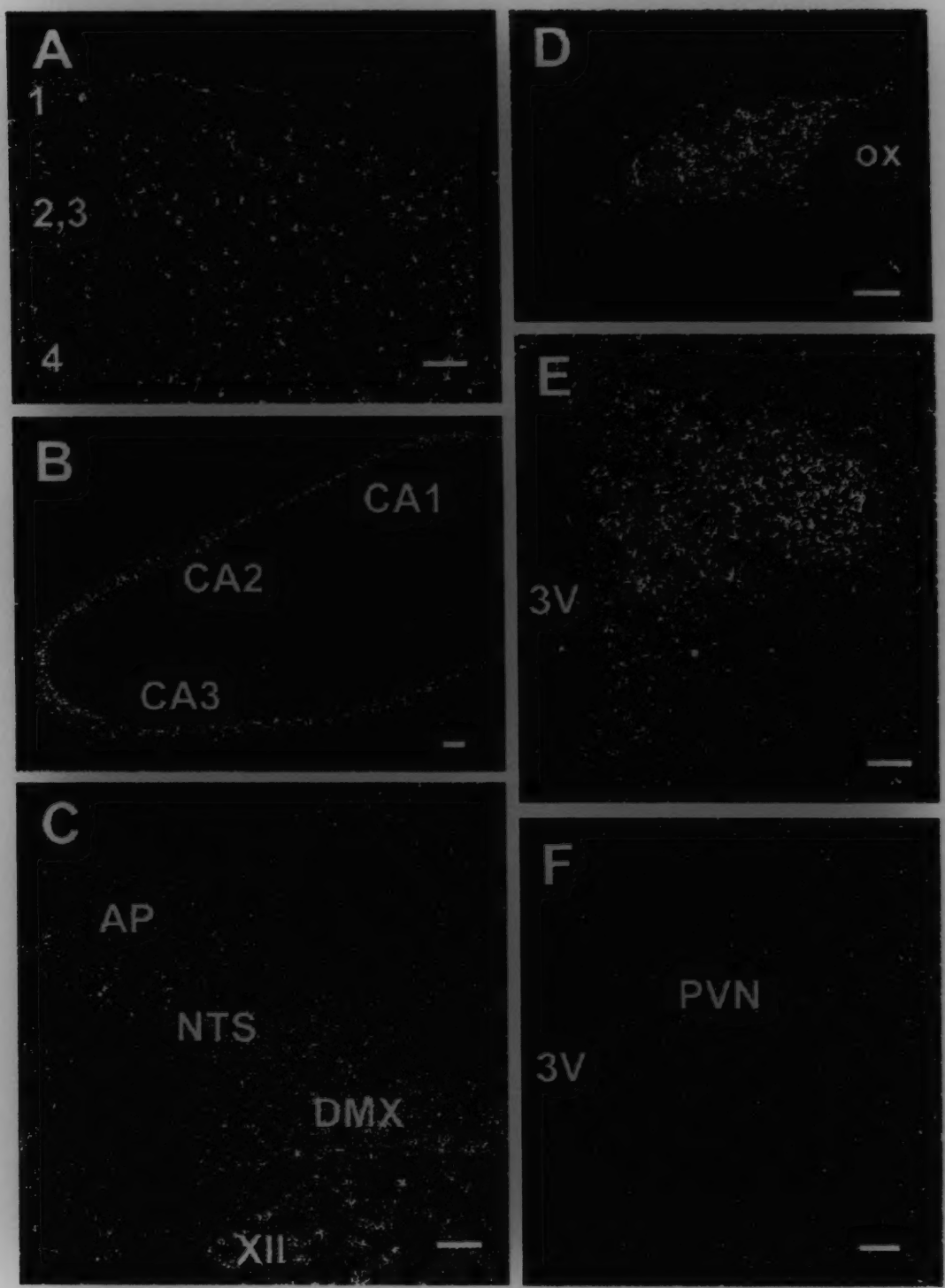
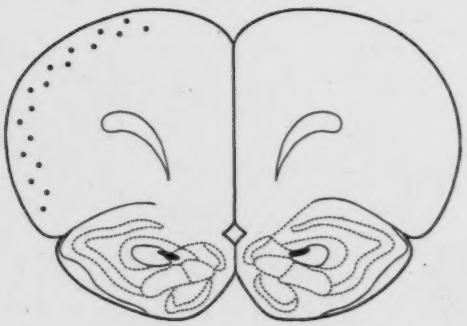
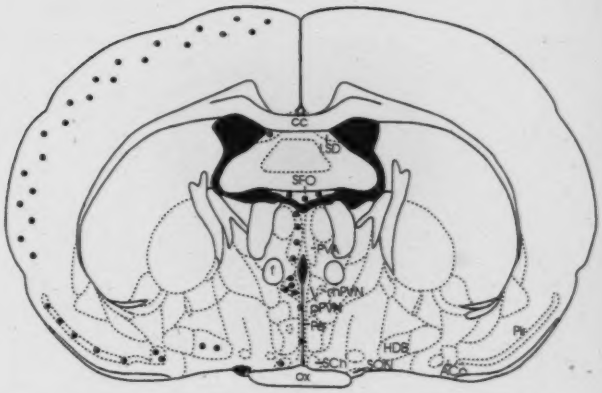

A
1
2,3
4
B
CA1
CA2
CA3
C
AP
NTS
DMX
XII
D
ox
E
3V
F
PVN
3V

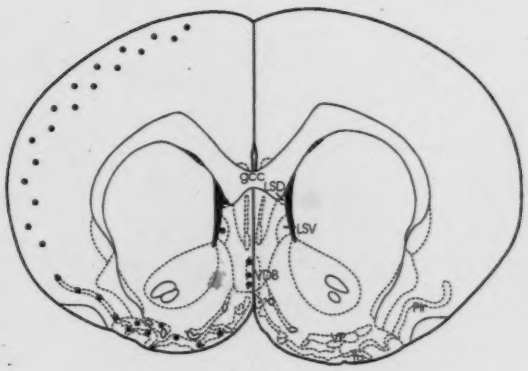
Figure 3.2: Schematic diagrams of coronal sections of rat brain illustrating the distribution of cells expressing RAMP-2 mRNA. The density of the dots represents the relative density of signal. Drawings were modified from the atlas of Paxinos and Watson (1986). For abbreviations, see list; numbers indicate distance from bregma. (Continued on next page)



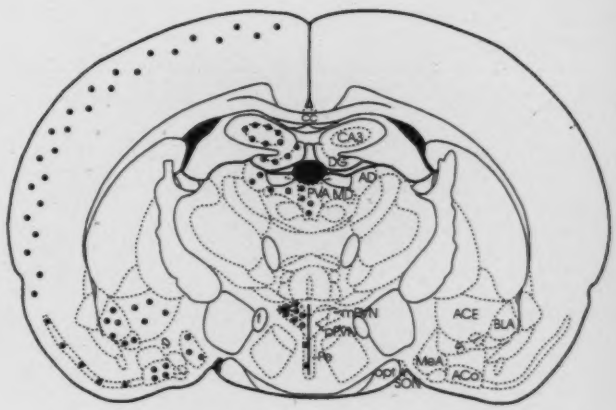
3.7 mm



-0.92 mm



1.6 mm



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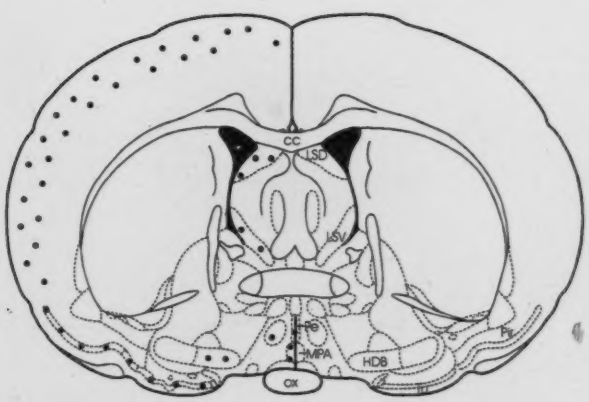

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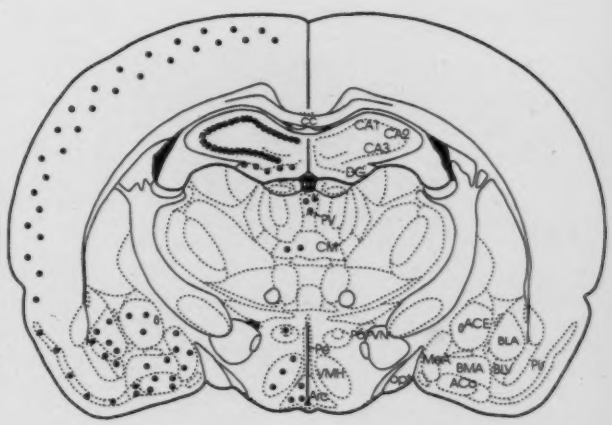

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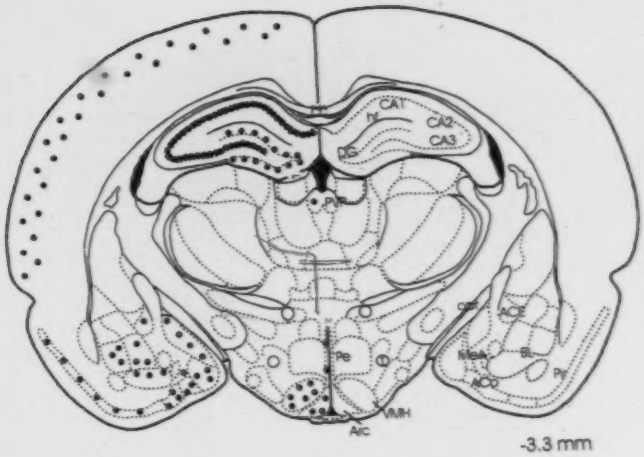

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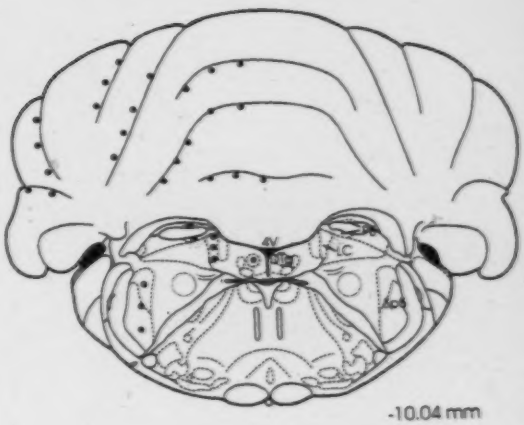

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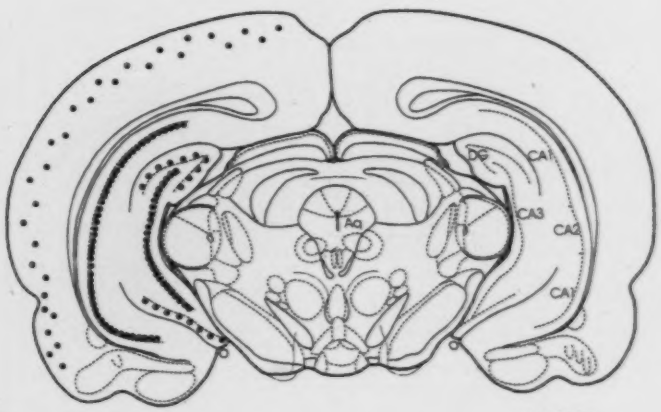

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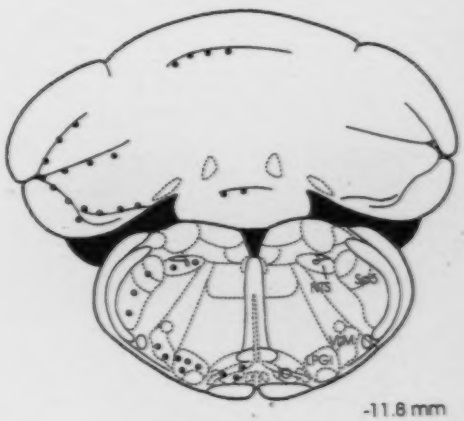

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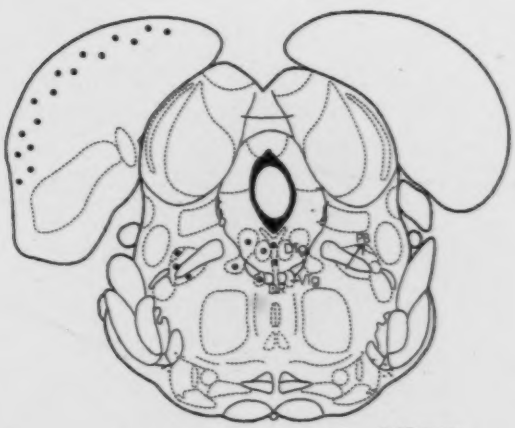

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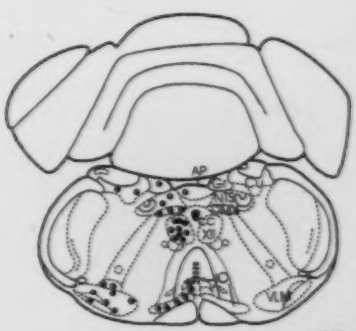

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Myelencephalon

High levels of signal were present in the hypoglossal nucleus, and moderate levels were seen in the ventrolateral medulla and paragigantocellular reticular nucleus. Low levels of signal were seen in the nucleus of the solitary tract, the area postrema, and the dorsal motor nucleus of the vagus, as well as the inferior olivary, the spinal trigeminal, gracile, and cuneate nuclei.

The posterior pituitary also showed low levels of RAMP-2 mRNA signal.

3.2 CRLR mRNA Distribution

ISH of CRLR revealed mRNA expression in the amygdaloid region (Fig. 3.3), primarily in neuronal cells as determined with cresyl violet counterstain. Little or no signal was detected elsewhere with ISH. In contrast, CRLR mRNA was detected by RT-PCR in every CNS area measured, including the amygdala, hypothalamus, brainstem, cerebellum, and spinal cord. The relative amounts of mRNA, normalized against an actin control, were high in the lung (the positive control), and relatively low in the amygdala, brainstem, cerebellum, hypothalamus, and spinal cord. The mRNA levels in the lung were significantly different from those of all other areas. Figure 3.4 shows the quantitation of a representative gel.

3.3 Mean Arterial Pressure Changes

Nitroprusside infusion caused a significant decrease in MAP (Fig. 3.5). Vehicle treated animals showed a slow, gradual decline in MAP over the 6 hours. Phenylephrine infusion caused a significant increase in MAP. Again, vehicle showed a slow decline in

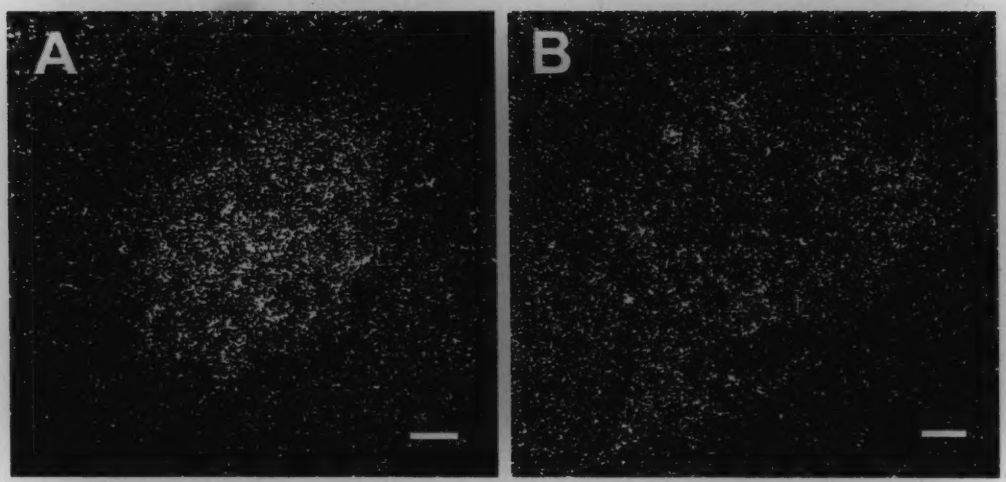


Figure 3.3: Dark field photomicrographs of similar sections from the basolateral amygdala, illustrating (A) RAMP-2 and (B) CRLR signal. Calibration bar = 100 μm.

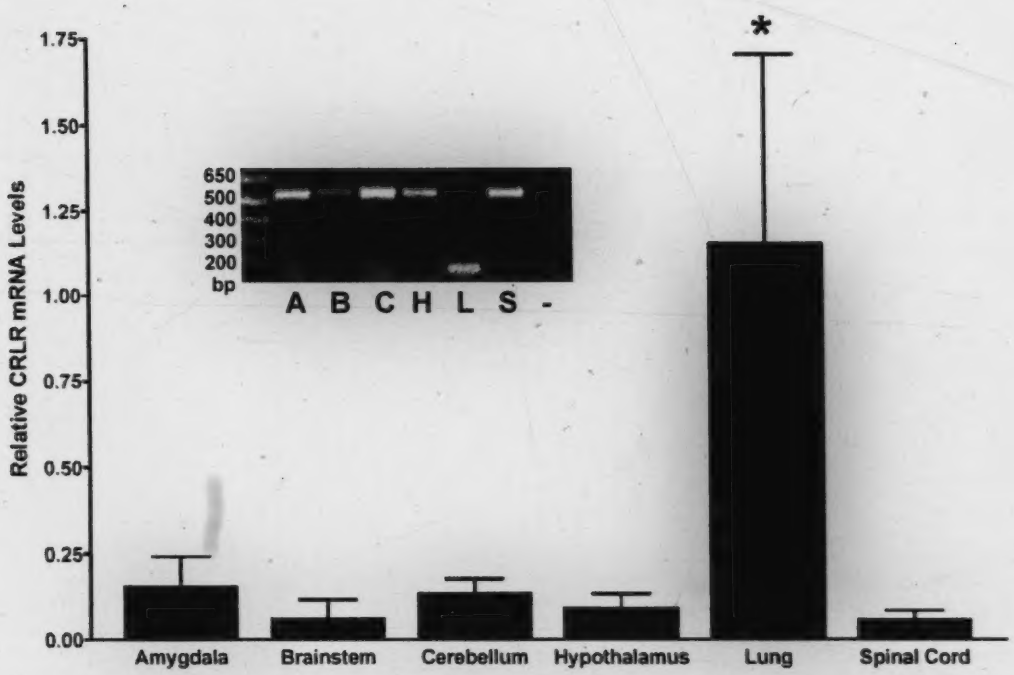


Figure 3.4: Relative levels of CRLR mRNA in various rat brain regions, detected by RT-PCR. Each value was normalized against a control (actin) band. The graph illustrates quantitation of one representative run. Inset: Ethidium bromide gel illustrating relative band intensities of actin (top band) and CRLR (bottom band) from amygdala, brainstem, cerebellum, hypothalamus, lung, and spinal cord (left to right). Size markers (in base pairs) are shown on the left. A control reaction, lacking the RT enzyme, is shown in the right lane (-). * indicates $p<0.05$ vs. all other areas.

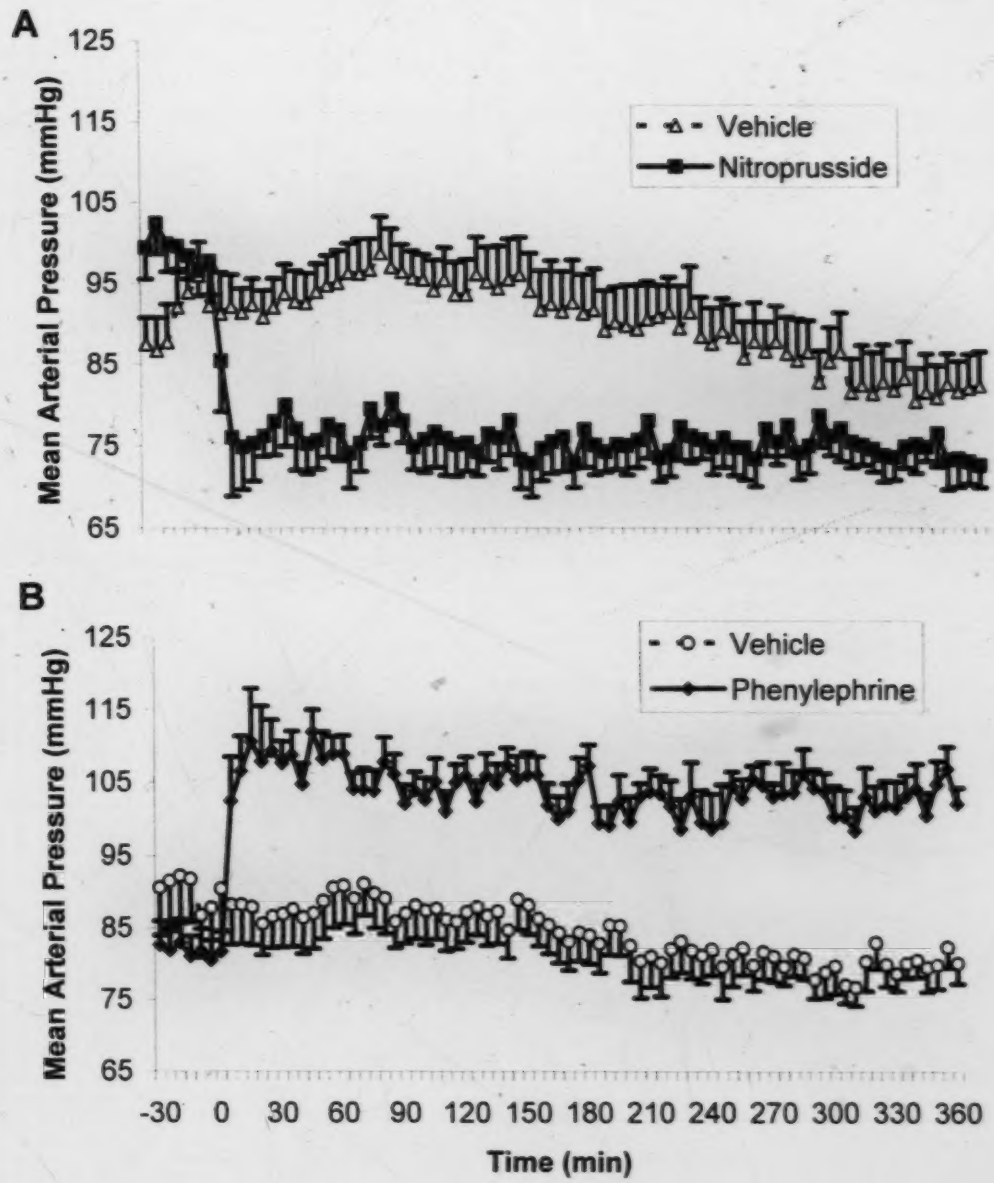


Figure 3.5: MAPs in nitroprusside and phenylephrine treated animals. (A) MAP in NP treated animals remains reduced for a 6-hour period compared to vehicle MAP. (B) MAP in PHENYL treated animals remains elevated for a 6-hour period compared to the vehicle control. Arrows indicate the start of infusion.

MAP over the 6 hours. Most of the rats fell asleep over the course of the experiment, which may account for the gradual reduction in MAP observed in vehicle treated rats.

3.4 Modulation of RAMP-2 and ppADM mRNA expression

Dark field photomicrographs illustrating examples of changes in RAMP-2 and ppADM mRNA signal are shown in Figure 3.6. RAMP-2 mRNA levels were increased in the middle NTS of NP treated animals, while ppADM mRNA levels were reduced in the pmPVN and mpvPVN subdivisions of PHENYL treated animals.

3.4.1 Effect of nitroprusside treatment on RAMP-2 mRNA

Nitroprusside treatment produced no significant differences in levels of RAMP-2 mRNA in forebrain areas compared to vehicle. In the brainstem, nitroprusside treatment produced a significant increase of +190 % in RAMP-2 mRNA signal in the middle NTS compared to vehicle treatment (Fig. 3.7).

3.4.2 Effect of phenylephrine treatment on RAMP-2 mRNA

Phenylephrine treatment resulted in a significant reduction in levels of RAMP-2 mRNA in the ventral medial parvocellular PVN (-46.0 %), as well as the rostral (-46.2 %) and caudal (-51.2 %) NTS compared to vehicle treated animals (Fig. 3.8).

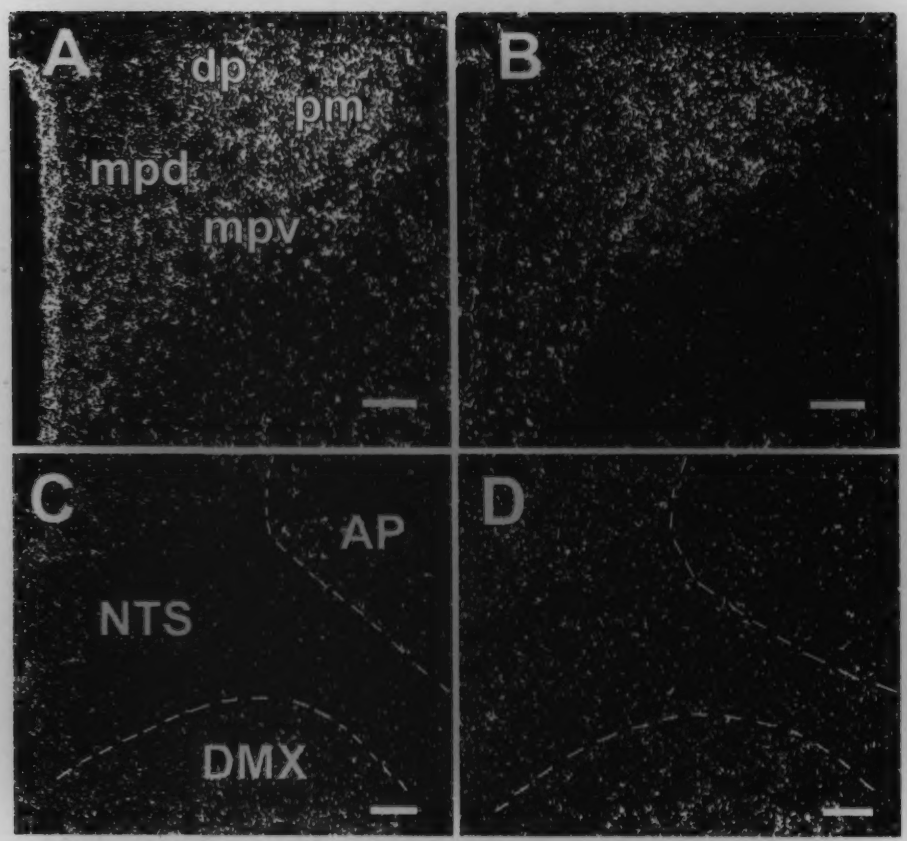
3.4.3 Effect of nitroprusside treatment on ppADM mRNA

Nitroprusside treatment produced a reduction in ppADM mRNA signal in the posterior magnocellular (-46.6 %), ventral medial parvocellular (-35.1 %), and lateral caudal parvocellular (-36.1 %) subdivisions of the PVN compared to controls. No significant differences were seen in brainstem areas in response to nitroprusside treatment (Fig. 3.9).

3.4.4 Effect of phenylephrine treatment on ppADM mRNA

Phenylephrine treatment produced no significant differences in levels of ppADM mRNA in either forebrain or brainstem areas (Fig. 3.10).

Figure 3.6: Photographs of ppADM and RAMP-2 ISH in NP, PHENYL, and control animals. (A,B) Levels of ppADM signal are reduced in the pm and mpv PVN subdivisions of NP treated animals (B) compared to control animals (A). (C,D) Levels of RAMP-2 signal are increased in the middle NTS of NP treated animals (D), compared to control animals (C). For abbreviations, see list. Calibration bars = 100μm.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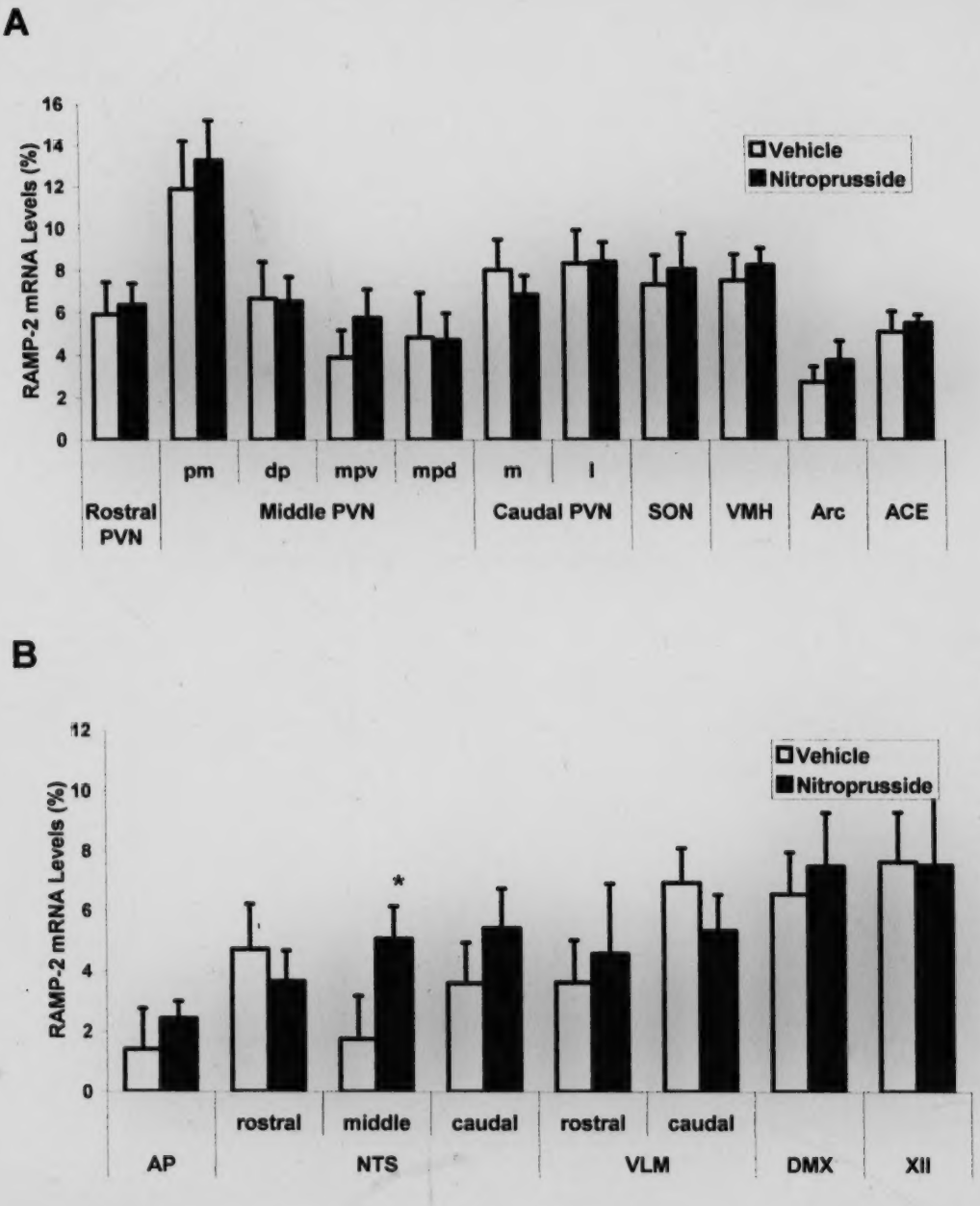

Figure 3.7: RAMP-2 mRNA signal in specific forebrain (A) and brainstem (B) nuclei in nitroprusside treated animals. NP animals show an increase in signal in the middle NTS. The signal is calculated as the percent of the area of the nucleus that is covered with silver grains, and is displayed as mean ± standard error.
* indicates $p<0.05$

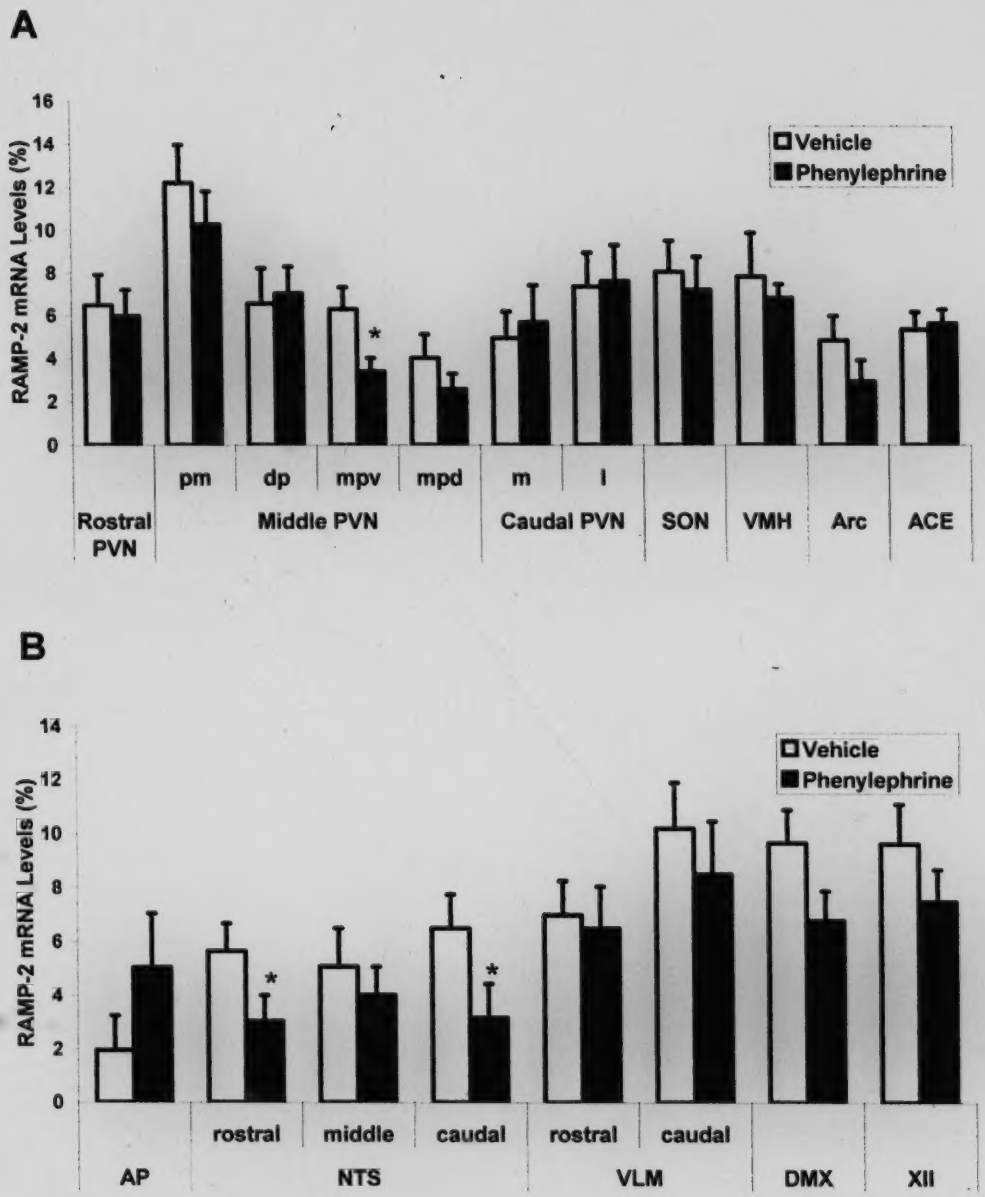

Figure 3.8: RAMP-2 mRNA signal in specific forebrain (A) and brainstem (B) nuclei in phenylephrine treated animals. PHENYL animals show a decrease in signal in the mpvPVN, and in the rostral and caudal NTS compared to controls.
* indicates $p<0.05$

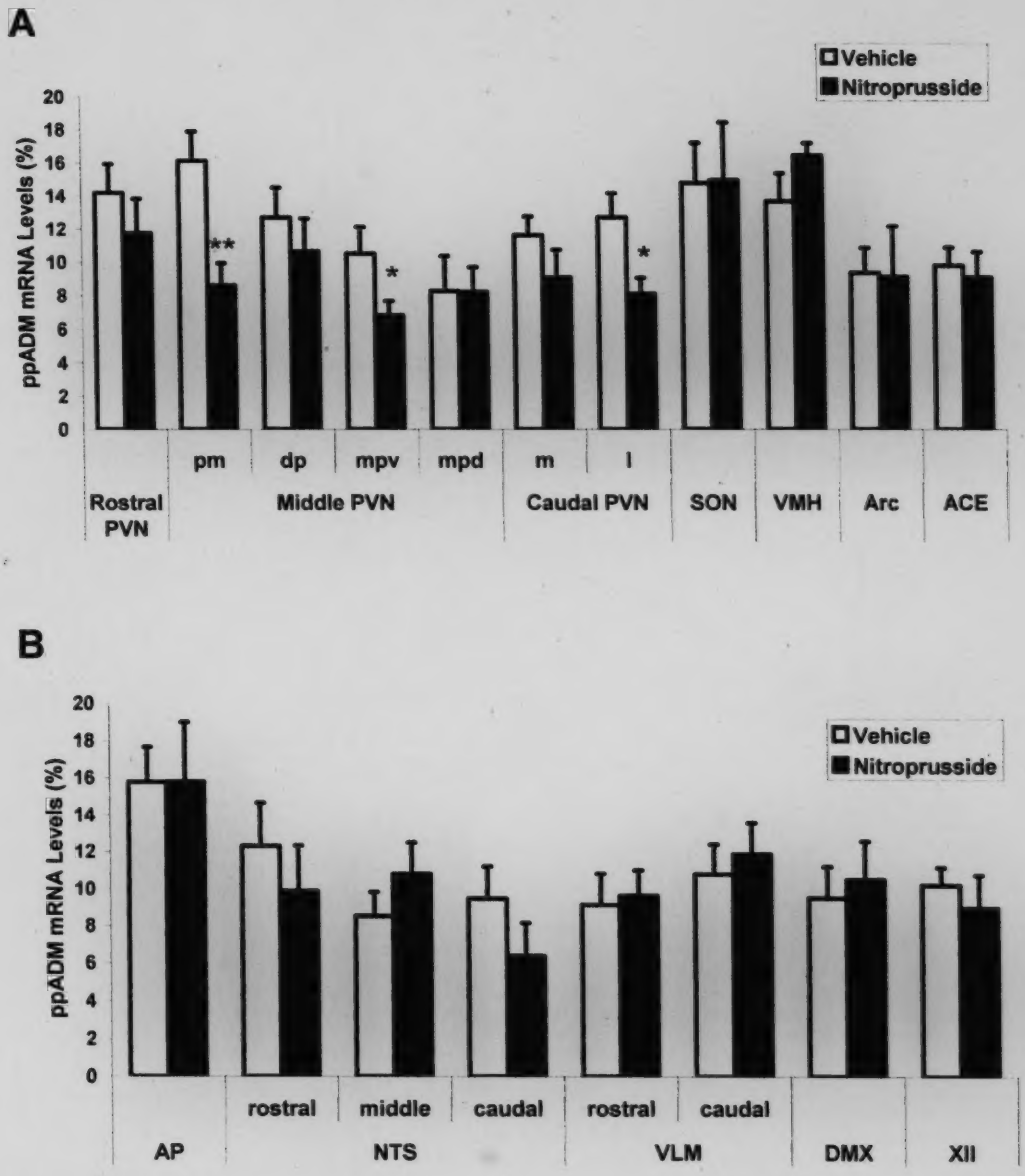

Figure 3.9: ppADM mRNA signal in specific forebrain (A) and brainstem (B) nuclei in nitroprusside treated animals. NP animals show a decrease in signal in the pmPVN, mpvPVN, and clPVN.
* indicates $p<0.05$, ** indicates $p<0.005$

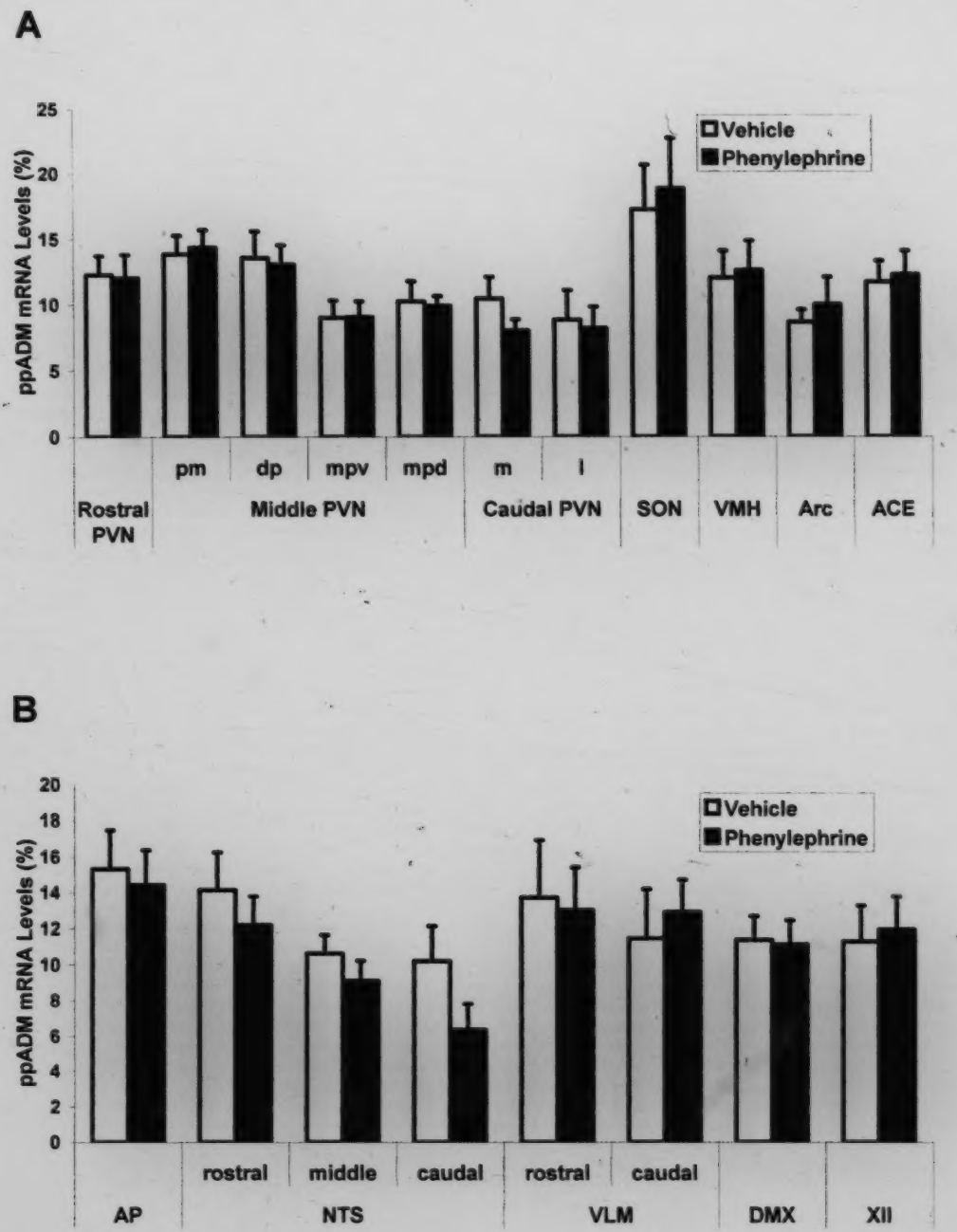

Figure 3.10: ppADM mRNA signal in specific forebrain (A) and brainstem (B) nuclei in phenylephrine treated animals. PHENYL animals show no change in signal compared to controls.
* indicates $p<0.05$

CHAPTER 4

DISCUSSION

4.1 CNS RAMP-2/CRLR Distribution

Our results show that RAMP-2 mRNA is found throughout the brain, and in numerous autonomic centers. The extensive distribution of RAMP-2 mRNA, along with the widespread but relatively low expression of CRLR mRNA suggests a role for the RAMP-2/CRLR ADM receptor in ANS function. Although previous data argue against the presence of the ADM receptor in most of the CNS, our results suggest an extensive expression in the CNS, particularly in autonomic centers. Our results help to resolve the apparent contradiction between ADM receptor distribution and the observed effects of ADM in the CNS.

RAMP-2 is widely accepted to be a component of an ADM receptor, along with CRLR. The results of this study show the CNS distribution of RAMP-2 mRNA, and may represent the CNS distribution of the RAMP-2/CRLR ADM receptor. However, there are two important caveats to take into account. First, the expression of mRNA is not necessarily indicative of protein expression, and second, the expression of one component of the RAMP-2/CRLR receptor does not necessarily confirm the presence of the receptor. With these caveats in mind, our results are suggestive of a wide-ranging distribution for an ADM receptor in the CNS.

The RAMP-2 distribution found in this study is similar to reports from Oliver et al. (2001), with some notable differences. For example, we found strong signal in the SON, PVN, and hypoglossal nuclei, moderate signal in the VLM, and low levels of signal in the PB, AP, DMX, locus coeruleus (LC), subfornical organ (SFO), purkinje cell layer of the cerebellum, and others which were not previously reported (see Table 3.1). The presence of RAMP-2 in these autonomic nuclei suggests a more extensive role for ADM

in CNS signaling than indicated by previous studies. Conversely, Oliver et al. (2001) reported strong signal in the dorsomedial hypothalamic nucleus, moderate signal in the facial nucleus and the substantia nigra, and weak signal in the granule cell layer of the cerebellum. We observed no detectable signal in these areas. The variations in reported signal are likely due to differences in specific activity of the probe, affinity of the probe, and incubation times. Furthermore, the control rats used in our experiment received infusions of vehicle. Although this treatment may have affected the distribution of RAMP-2 mRNA, this possibility seems unlikely as pilot studies in our lab using untreated rats showed similar results. Finally, RAMP-2 expression is low in many CNS nuclei; this weak signal makes detection difficult and may account for some of the differences between our results and those of Oliver et al. (2001).

RAMP-2 is expressed in many autonomic nuclei, including PVN, NTS, AP, VLM, PB, ACE, and SON. Since these areas show extensive interconnections (Calaresu et al., 1984), the fact that they express both RAMP-2 and ppADM suggests that ADM is produced by, and can act upon these centers. This finding is consistent with the effects of ADM in the CNS, both in terms of the activation of neurons by ICV injection of ADM, and the physiological effects of ADM in ICV and microinjection experiments. For example, ICV ADM was shown to activate neurons in the NTS, ACE, VMH, Arc, and SON (Ueta et al., 2001; Shan and Krukoff, 2001b). Since no previous studies showed ADM receptor expression in these areas, it was not known whether ICV ADM could act directly on these nuclei, or only activated them indirectly through blood pressure changes. Although our results do not resolve the issue of direct versus indirect activation by ADM, they do suggest that direct activation is possible. In addition, expression of

RAMP-2 in the PVN and AP is consistent with experiments showing that ADM can cause changes in blood pressure when injected to these nuclei (Allen et al., 1997; Smith and Ferguson, 2001).

Central autonomic nuclei, including the Arc, PVN, and VMH, have been implicated in feeding behavior (Schwartz et al., 2000). The presence of RAMP-2 in these nuclei suggests that ADM may influence feeding behavior by acting at these locations. Likewise, since the NTS, AP, SFO, SON, PVN, and hypothalamic preoptic areas have been implicated in drinking behavior (Sewards and Sewards, 2000), the presence of RAMP-2 suggests that ADM may exert its reported effects on drinking behavior and salt appetite by acting on these nuclei.

Our CRLR ISH results show that CRLR expression is confined to the amygdaloid region in the CNS, consistent with previous reports of CRLR mRNA distribution (Fluhmann et al., 1997; Oliver et al., 1998). In contrast, our RT-PCR results suggest that CRLR is expressed at a low level in many other brain areas. The differing results of the two techniques indicate that ISH may not be sensitive enough to detect CRLR mRNA. Indeed, the inability to detect mRNA for CRLR does not exclude the presence of CRLR protein, as observed by Choksi et al. (2002) in L6 myoblasts. By using RT-PCR, a more sensitive measure of mRNA levels, we were able to detect CRLR in the amygdala, hypothalamus, brainstem, cerebellum, and spinal cord of rats. As the use of tissue blocks prevents specific localization of mRNA, we cannot determine whether the CRLR is produced in neuronal, glial, vascular, or other cell types. However, as the CRLR ISH signal is primarily localized to neurons in the amygdala, a similar distribution may occur in other brain regions. Using semi-quantitative analysis, we found that the mRNA levels

were relatively low in the hypothalamus and brainstem, which may account for our inability to detect CRLR in these areas using *in situ* hybridization. Alternatively, a more dense distribution of CRLR mRNA in the amygdala compared to other regions may account for the ability to detect signal in this region, even if the overall mRNA levels are not significantly different from other CNS regions.

The presence of RAMP-2, and low levels of CRLR mRNA throughout the CNS suggest that the RAMP-2/CRLR receptor may be expressed at low levels in many autonomic areas. Low expression levels may explain why autoradiographic binding studies showed ADM binding sites in the amygdaloid region, but none in the hypothalamus and brainstem (Juaneda et al., 2001). Receptor expression may be below the limits of detection of autoradiographic binding, with the exception of the amygdaloid region, which expresses higher levels of CRLR, as shown by ISH (Oliver et al., 2001). It has been observed that the third component of the RAMP-2/CRLR receptor, RCP, is widely expressed in the rodent CNS (Oliver et al., 1999), including many of the areas expressing RAMP-2. Therefore, the three components of an ADM receptor complex can all be detected in many brain areas, consistent with the idea of widespread expression of low levels of RAMP-2/CRLR receptor.

4.2 RAMP-2 mRNA Levels are Altered in Response to Changes in MAP

In order to observe changes in mRNA, it is necessary to maintain a stimulus long enough to allow gene transcription to occur. Previous studies indicate that a stimulus of 4 hours is sufficient to cause changes in expression of some genes (Ono et al., 2000; Shan and Krukoff, 2001a). However, changes in mRNA levels may occur with different time

courses, as illustrated in vascular smooth muscle cells, where dexamethasone treatment produced a maximal change in RAMP-1 at 4 hours, as opposed to a maximal change in ppADM at 8 hours (Frayon et al., 2000). Likewise, sepsis induced a maximal change in RAMP-2 mRNA at 4 hours (Ono et al., 2000). We therefore used a 6 hour stimulus (change in MAP) to examine effects on RAMP-2 and ppADM mRNA levels. While we saw changes in both RAMP-2 and ppADM mRNA after 6 hours of altered MAP, it is possible that the maximal changes in mRNA levels occur at a time other than 6 hours.

Blood pressure alterations resulted in changes in RAMP-2 mRNA levels and therefore suggest that changes in ADM receptor expression occur in relation to changes in MAP. Autelitano and Ridings (2001) showed that in cardiomyocytes, increasing expression of either RAMP-2 or CRLR resulted in an up-regulation of ADM receptor, while increasing expression of both led to a further increase. Since an increase in the amount of either component can drive an increase in receptor formation, the production of the receptor from its component parts is likely a dynamic process. This theory supports our assumption that changes in RAMP-2 expression are indicative of changes in receptor expression. The observed alterations in RAMP-2 mRNA levels suggest that the central ADM system is modulated in response to MAP changes and that brain ADM has a role in regulating blood pressure.

RAMP-2 mRNA levels in the brain tended to decrease in response to MAP increases and increase in response to MAP decreases, in agreement with findings in other tissues. For example, a negative correlation exists between blood pressure and RAMP-2 mRNA expression in the umbilical artery and uterus of patients with pregnancy induced

hypertension (Makino et al., 2001). In congestive heart failure, blood pressure was significantly lowered, while cardiac RAMP-2 was up regulated (Totsune et al., 2000).

The specific nuclei showing changes in mRNA were the NTS and PVN, which play a role in cardiovascular, autonomic, and endocrine integration (Benarroch, 1997). In the NTS, RAMP-2 mRNA was increased in response to decreased MAP, and decreased in response to increased MAP. The reciprocal changes in RAMP-2 suggest a physiological role for the RAMP-2/CRLR receptor in the regulation of blood pressure. Since the primary role of the NTS in the baroreflex is sensory, the physiological actions of ADM in the NTS may only be effective when the system is perturbed. In anesthetized animals, ADM microinjection into the NTS had no effect on MAP (Allen et al., 1997). Similarly, blocking NO production in the NTS did not alter MAP under control conditions (Lin et al., 1999). However, when the NTS was first stimulated with glutamate, blocking NO production attenuated the drop in MAP (Lin et al., 1999). As NO often acts as a mediator of the effects of ADM (Hinson et al., 2000), a similar perturbation may be necessary to observe the effects of ADM in the NTS. Here, we have perturbed blood pressure, and observed a reciprocal change in RAMP-2, suggesting that ADM may act in the NTS to influence blood pressure homeostasis.

In animals with elevated blood pressure, RAMP-2 mRNA was reduced in the mpvPVN. The mpvPVN sends projections to brainstem and spinal cord to regulate sympathetic drive (Sawchenko and Swanson, 1982; Calaresu et al., 1984). RAMP-2 alterations in the PVN will affect the ability of central ADM to regulate sympathetic drive. However, the specific effects of ADM in the mpvPVN are difficult to predict. ADM microinjection to the PVN decreased blood pressure (Smith and Ferguson, 2001),

while microstimulation of the PVN caused either increases or decreases in MAP (Yamashita et al., 1987; Kannan et al., 1989; Martin and Haywood, 1992), depending on whether the site of injection is parvocellular or magnocellular (Porter and Brody, 1986). Since ADM can both stimulate and inhibit neurons (Allen and Ferguson, 1996), it is not clear whether ADM will increase or decrease MAP when acting in the mpvPVN. Thus, more study is needed to determine the effects and mechanisms of action of ADM in specific subnuclei of the PVN, in order to determine what effects the observed decrease in RAMP-2 may have on homeostatic function.

4.3 ppADM mRNA Levels are Reduced in Response to a Reduction in MAP

CNS ppADM mRNA levels are reduced in hypotensive rats, in agreement with the observations of Shan and Krukoff (2001a), who found that LPS treatment, which reduces blood pressure, also reduces ppADM mRNA in the CNS. The reductions in ppADM mRNA occurred in the pmPVN, the mpvPVN, and clPVN.

Neurons of the pmPVN secrete OXY and AVP from the posterior pituitary. The presence of ppADM mRNA in the pmPVN implicates ADM in the regulation of OXY and AVP secretion. The observation that RAMP-2 mRNA is present in the posterior pituitary suggests that ADM could act at the level of the posterior pituitary to regulate AVP and OXY. AVP secretion plays an essential role in maintaining blood pressure, especially under hypovolemic conditions (Bennett and Gardiner, 1985), and may therefore be influenced by the observed reductions in ppADM. Although it is not clear what effects ADM may have on AVP in the posterior pituitary, central ADM has been shown to inhibit stimulated AVP release (Yokoi et al., 1996). The observed down-

regulation of ADM in the pmPVN may therefore act to disinhibit AVP secretion in hypotensive rats, in order to help restore blood pressure.

A reduction in ppADM mRNA also occured in the mpvPVN and clPVN of animals with reduced MAP. Alterations in ppADM in response to low blood pressure suggest a homeostatic regulation of ADM synthesis. A down-regulation of ppADM in response to hypotension would be homeostatically favorable if the ADM produced in mpvPVN and clPVN normally acts to decrease blood pressure. Indeed, ADM acts in the PVN to decrease blood pressure (Smith and Ferguson, 2001). Combined with the observation that some parvocellular neurons send collaterals back into the PVN to produce recurrent connections (Armstrong, 1995), these data suggest that ADM may act in a paracrine/autocrine manner in the PVN. If such is the case, then down-regulating ADM in the PVN of hypotensive animals would help to restore blood pressure.

5 Conclusions

ADM is a peptide with a wide variety of actions in the CNS and periphery. The CNS distribution of RAMP-2, a component of an ADM receptor, is consistent with the role of ADM in autonomic regulation. Autonomic nuclei that regulate blood pressure, eating, and drinking express RAMP-2 mRNA. The distribution of RAMP-2 provides an anatomical basis for many of the observed CNS effects of ADM. RAMP-2 distribution likely represents the distribution of the RAMP-2/CRLR receptor, particularly in view of the widespread detection of CRLR mRNA in the CNS. The observed alterations in the CNS expression of RAMP-2 and ppADM mRNA in the PVN and NTS, important nuclei

in the autonomic regulation of blood pressure, in response to changes in blood pressure are consistent with a role for ADM in re-establishing cardiovascular homeostasis.

This study suggests the need for future experiments. The alterations of ADM signaling components in the PVN are difficult to interpret, due to a lack of information regarding the specific roles of PVN subdivisions in blood pressure, and the actions of ADM in these subdivisions. To resolve these issues, studies using microinjections of ADM to specific subdivisions of the PVN would be informative. Additionally, the development of specific antagonists for ADM will help to clarify the effects of endogenous ADM in autonomic nuclei.

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